CE LIBRARY

JUL 30 1923

BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

VOL. XLIV JUNE, 1923 NO. 6

CONTENTS

HOADLEY, LEIGH. *Certain Effects of the Salts of the Heavy Metals on the Fertilization Reaction in Arbacia punctulata* 255

WATT, JAMES C. *The Behavior of Calcium Phosphate and Calcium Carbonate (Bone Salts) Precipitated in Various Media, with Applications to Bone Formation* . . 280

PUBLISHED MONTHLY BY THE

MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY

LANCASTER PRESS, INC.

LANCASTER, PA.

AGENT FOR GREAT BRITAIN

WHELDON & WESLEY, LIMITED

2, 3 and 4 Arthur Street, New Oxford Street, London, W. P. 2

Single Numbers, 75 Cents. Per Volume (6 numbers), $3.00

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16 1894.

Editorial Staff

E. G. CONKLIN—*Princeton University.*

GEORGE T. MOORE—*The Missouri Botanic Garden.*

T. H. MORGAN—*Columbia University.*

W. M. WHEELER—*Harvard University.*

E. B. WILSON—*Columbia University.*

Managing Editor

FRANK R. LILLIE—*The University of Chicago.*

All communications and manuscripts should be sent to the Managing Editor, the University of Chicago, Sept. 15th to June 15th, or Woods Hole, Mass., June 15th to Sept. 15th. Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa.

Vol. XLIV. *June, 1923.* *No. 6.*

BIOLOGICAL BULLETIN

CERTAIN EFFECTS OF THE SALTS OF THE HEAVY METALS ON THE FERTILIZATION REACTION IN *ARBACIA PUNCTULATA*.

LEIGH HOADLEY,

THE HULL ZOÖLOGICAL LABORATORY, UNIVERSITY OF CHICAGO.

I. Introduction..... 255
II. Normal fertilization reaction in *Arbacia*..... 258
III. Methods..... 258
IV. Experiments..... 260
a. Data on various metals..... 260
b. Comparative tests..... 271
c. Summary..... 274
V. Discussion..... 275
VI. Conclusions..... 277

I. INTRODUCTION.

At the suggestion of Prof. F. R. Lillie in the spring of 1921, I began, the following summer, a study of the effects of the heavy metals on the fertilization reaction in *Arbacia punctulata*. I desire here to express my appreciation for his many helpful suggestions and his aid in the interpretation of the results.

Lillie ('21) found that copper salts completely prevent the elevation of fertilization membranes in *Arbacia* even at such a great dilution as 1 : 500,000, though the concentration of copper chloride required for the suppression of cleavage is 1 : 62,500. It is the purpose of the present set of tests to establish the effects of a series of metallic salt solutions on the fertilization reaction and by this means to throw more light on the initial events of this reaction. The work was done during the summers of 1921 and 1922 at the Marine Biological Laboratory at Woods Hole, Massachusetts.

The experiments brought out very clearly that there is a sharp distinction between the initial and the subsequent events

of the fertilization reaction. As soon as the initial part of the reaction is completed, *i.e.*, as soon as the membrane has raised, the metallic concentration which has formerly been inhibitory in its action ceases to be wholly effective and some of the eggs may develop as far as the motile free-swimming forms in the solution. In the following pages, such a concentration, which inhibits the elevation of membranes, when eggs are inseminated in it, will be termed "membrane-inhibitory." Such inhibition of membrane elevation is complete. If, however, the concentration of the salt is greatly increased, a concentration may be reached at which cleavage in turn is completely inhibited, but here again, the time factor is important. For example, if a given concentration is toxic to cleavage for a set of eggs transferred to it five minutes after insemination in normal sea water, it is said to be "cleavage-toxic." It may allow 40 per cent. to 50 per cent. cleavage if eggs are transferred to it 20 minutes after such an insemination, or even 95 per cent. if they are transferred after a 30-minute interval. In such a case, second cleavage will be completely inhibited. When cleavage is used as a criterion of toxicity, the period of exposure to the solution will vary according to the length of time elapsing between insemination and transfer. If, when eggs are transferred to the solution five minutes after insemination, first cleavage is inhibited, then it follows that eggs transferred at a later time will not perform second cleavage, though they may cleave once.

McGuigan ('04), v. Euler and Swanberg ('21), Olsson ('21), and others have investigated the effects of the salts of certain of the heavy metals on enzyme action. Mathews ('04), working with *Fundulus*, compares the physiological action of the heavy metals with their solution tensions. It was with the same idea that McGuigan worked on the ferments. He found that, generally speaking, a low solution tension was indicative of great toxicity. There were, however, certain exceptions to the series. v. Euler and Swanberg attribute the poisonous effect of the salts to their property of binding certain groups of the enzyme molecule and thus making it ineffective.

In the ferment experiments just cited as well as in the experiments made by Mathews on *Fundulus*, the experiments allowed

the use of distilled water in the solutions, and in consequence, the dissociation of the salts could be accurately measured and precipitation did not trouble the investigators. In the work with *Arbacia*, on the other hand, it was necessary to use sea-water for all of the final solutions though the stock solutions were made in distilled water. The composition of the sea-water is of such a nature that in many cases there is a precipitation and in no case could the ionization of the salt be accurately determined. The sea-water, as it comes from the tap in the laboratory, has a p.H. of 8.0 and I may say here, that in no case did the concentration of the metallic salt necessary to inhibit membrane formation have a p.H. below 7.0. In some of the solutions there was a visible precipitate, as was most evident in the cases of Zinc, Lanthanum, and Lead. In such solutions, the balance of the salts must be disturbed. Thus, in the case of the alkaline earth metals, and silver, tin, manganese, chromium, and iron, the precipitate was so heavy that after a few preliminary experiments, their use was abandoned. The precipitation of zinc, lanthanum, and lead was not great enough to hide the effect of the salts on the elevation of the membranes but when the concentration was increased for the investigation of cleavage-toxicity, the precipitation was so heavy that it was deemed unwise to try to determine that point for them.

When the series was completed, it was found that the work included tests made with the following metals: Gold, copper, zinc, lanthanum, aluminum, platinum, lead, nickel, cadmium, cobalt, and mercury. The results of the experiments show a similar behav or of the metals, save mercury, and those described by Lillie ('21) for the chloride of copper. The inhibiting concentration varies for the different metals.

The behavior of mercuric salts is peculiar and unexplainable at present. Its solutions seem to favor membrane elevation up to a concentration at which the sperm are immediately paralyzed, while at a much greater dilution, cleavage is completely inhibited. This will be treated in more detail in the following pages.

Rhubidium and cesium were found to be indifferent up to the isotonic concentration and will not be treated further.

II. The Normal Fertilization Reaction in *Arbacia Punctulata*.

In order to understand the later discussion, it will be wise here to summarize the phenomena of the normal fertilization reaction in *Arbacia*. These were described in great detail by Lillie ('14), though some of the events had been incompletely described by von Dungern ('02). Fol ('79) described the penetration of the spermatozoön and the elevation of the fertilization membrane in a classical monograph.

Before the spermatozoön comes into contact with the egg itself it is activated and directed toward the egg as described by Lillie ('13). The egg, from the time of rupture of the germinal vesicle up to the time of fertilization, or, when fertilization is barred, up to the time of cytolysis, produces an iso-agglutinin (Lillie '13) or fertilizin (Lillie '14) which produces a reversible agglutination of the spermatozoa to the egg. Between the time of the agglutination of the spermatozoa to the egg and the beginning of the fertilization reaction, there is a latent period which is very short but variable in *Arbacia*. This is followed by a very rapid initial reaction which produces a sterilization of the egg against other spermatozoa, described by Just ('19) as "a wave of negativity." The penetration of the spermotozoön occurs immediately, followed by the elevation of the membrane. In a given lot of fresh eggs, the time elapsing before membrane elevation is very variable. At 19.5° C. some eggs appear with membranes fully formed in from 15 to 30 seconds while others require much longer. Elevation of the membrane is too rapid for observation in *Arbacia* and it seems probable that the variable is due to the latent period. With stale gametes the process may extend over five minutes or more and is often accompanied by polyspermy.

III. Methods.

The experiments described in the following pages are very simple. Eggs were removed from good ripe females by making a circumferential cut around the animal and removing the gonads intact to about 150 c.c. of sea water in a finger bowl. The ovaries were then cut a number of times and the ova allowed

to stream forth. In about five minutes, the suspension was strained through cheese cloth to remove the pieces of the ovaries. The eggs were then allowed to settle. When they had settled, the supernatant liquid was poured off and the dish again filled with fresh sea water. This process was repeated three times so that all traces of the coelomic fluid and the tissue juices were practically eliminated. Eggs were not used unless the normally fertilized control showed above 95 per cent. membrane formation and a good viability through the first cleavage.

Males were cut in the same way and placed in a Syracuse glass with the genital pores down. The sperm flows from the pores in a creamy mass, which, when mixed with sea water in the proportion of one drop of dry sperm to 25 c.c. of sea water gives a grayish, opalescent suspension which is described in this paper as a 1 : 25 sperm suspension. One drop of this mixture when added to one drop of eggs in 7.5 c.c. of sea water gives 100 per cent. membrane formation with no polyspermy and such an insemination is spoken of as a 1 : 25 : 7.5. insemination.

Concentrated stock solutions were made up as percentage of metallic salt in distilled water by means of the volumetric flask. The solutions used in the experiments were made up by the addition of the stock solution to sea water. Care was taken with those metals which required high concentrations, that the proportion of sea water and stock solution in the mixture used should not alter the osmotic pressure to any appreciable extent. Solutions were not used for more than five days save in the case of $AuCl_3$ and $PtCl_4$ where the rarity of the metal demanded the use of the one solution.

When an insemination is spoken of as immediate, *i.e.*, made in the solution itself, 7.5 c.c. of the solution was put into a watch crystal and one drop of eggs and one drop of 1 : 25 sperm suspension added at opposite sides of the dish. The dish was immediately rotated to insure thorough mixing of the gametes. In the viability tests, the eggs were normally inseminated in sea water and one drop of these was then added to 7.5 c.c. of the solution. The dish was then rotated as stated for the immediate inseminations.

A number of comparative tests were made in which, owing to

the size of the experiment set up, it was necessary to use the eggs of a number of females. Unless so stated, each experiment was made with the eggs and the sperm of but one pair of individuals.

IV. Experiments.

I have chosen to deal with the experimental section of this paper in three parts. In the first I will take up in some detail the behavior of each solution which I used. I have taken the metals up in the order of decreasing toxicity. It will be noted that Mercuric chloride is apparently out of place, but as it gives no membrane inhibiting point in the same sense as the other metallic salts, it has been put at the end.

The series of tests described in part *b* was made for the purpose of comparison. The tests show the great similarity in the action of the various salt solutions on the eggs and the sperm, not only when they are unmixed, but also on insemination. The latter includes both immediate inseminations and viability tests.

Under *c* I have summarized the experimental data. There is also a table there that shows both the membrane-inhibiting concentration and the cleavage-toxic concentration for each of the metals.

a. Data on the Various Metallic Salts.

1. *Gold Chloride.*—On July 20, 1922, a 1 per cent. stock solution of $AuCl_3$ was made in distilled water and used for establishing concentrations in sea-water as shown in Table I*a*. These were prepared separately for, and at the time of each experiment. A series of tests was then set up to establish the effects of various concentrations on eggs which were inseminated immediately in the solutions. The results are shown in the accompanying Table I*a*. It can be seen on examination, that one part of $AuCl_3$ to 1,500,000 parts of sea-water will completely inhibit membrane elevation. At 1 : 600,000 and 1 : 300,000, narrow hyaline zones appear around the eggs after an exposure of about five minutes to the solution, but upon examining the control, it was found to be merely the first indicium of cytolysis in the egg.

A series of tests was then made with the purpose of determining what concentration is necessary to inhibit cleavage when the eggs are transferred to the solutions five minutes after insemina-

TABLE I*a*.

Concentration of $AuCl_3$ in Sea-water.	Per Cent. Membranes.		Per Cent. 1st Cleavage.	
	♀ *A*.	♀ *B*.	♀ *A*.	♀ *B*.
1 : 12,000,000	100	100	99	100
1 : 6,000,000	68	20	69	18
1 : 3,000,000	22	2	20	2
1 : 1,500,000	0	0	0	0
1 : 1,200,000	0	0	0	0
1 : 600,000	0	0	0	0
1 : 300,000	0	0	0	0
Uninseminated control in sea-water	0	0	0	0
Inseminated control in sea-water	100	100	100	100

Effect of $AuCl_3$ on eggs inseminated therein. July 20, 1922. Temp. 22.5° C. Eggs and sperm fresh. Insemination immediate 1 : 25 : 7.5.

tion in normal sea-water. The results for one of these tests is given in Table I*b*. Table I*b* shows the concentration of $AuCl_3$ toxic to cleavage to be 1 : 375,000. Comparison with Table I*a* brings out the difference between membrane-inhibiting and cleavage-toxic concentrations.

TABLE I*b*.

Concentration of $AuCl_3$ in Sea-water.	Per Cent. 1st Cleavage.	Per Cent. 2d Cleavage.
1 : 1,500,000	90	3
1 : 750,000	12	0
1 : 375,000	0	0
1 : 185,000	0	0
Inseminated control in sea-water	98	97

Test of cleavage-toxicity of $AuCl_3$. July 20, '22. Eggs from ♀ *A*. Opened 1 : 34 P.M. Membranes 100 per cent. Eggs transferred to solution five minutes after insemination in normal sea-water. 1 : 25 : 7.5. Sperm fresh. Temp. 24° C.

In Table I*c* can be seen viability tests run with the same lot of eggs at both the cleavage-toxic and the membrane-inhibitory concentrations. This test shows the difference between these two kinds of action. A concentration that is completely inhibitory to the initial events has no absolute effect on the subsequent events, while a more concentrated solution will prevent the occurrence of the subsequent events provided the time factor is sufficient. The effect of the exposure time is also shown by this table. The effect on cleavage is cumulative. The higher the concentration of the metallic salt, the shorter need the time of exposure be, to produce complete inhibition.

TABLE Ic.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.		Per Cent. 2 Cell.		Per Cent. 4 Cell.	
	A.	*B.*	*A.*	*B.*	*A.*	*B.*
Immediate	0	0	0	0	0	0
30 sec.	92	93	29	0	12	0
1 min.	95	95	46	0	14	0
2 "	98	98	80	2	65	0
4 "	100	100	84	4	68	0
8 "	100	100	84	16	80	0
15 "	100	100	92	24	86	0
30 "	100	100	96	78	95	0
Inseminated control in sea-water	100		98		99	

Viability tests. Aug. 12, '22. 5 : 27 P.M. Temp. 21° C. Eggs ♀ *B*. 5 : 10 P.M. Sperm fresh. Insemination 1 : 25 : 7.5. Solutions:

A, 1 : 1,500,000 $AuCl_3$.

B, 1 : 375,000 $AuCl_3$.

Lillie ('21) found that there are three ways in which egg when placed in the membrane-inhibiting concentration of $CuCl_2$ can be made to fertilize and produce membranes. Two of these are by means of protective agents. In the first case he used egg sea-water of high agglutinating power. In the second, he used a solution of gelatine or of gum arabic. The third method is by the use of an excess of sperm. A test was made of the effect of increased sperm concentrations on gold inhibition with the results shown in Table I*d*. A concentration of $AuCl_3$ of

TABLE Id.

Drops 1 : 25 Sperm.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1	0	0
2	0	0
4	0	0
6	4	3
8	20	20
12	36	34
20	100	92 much polyspermy.

Effect of increased sperm concentration. Aug. 7, 1922. 10 : 38 A.M. Temp. 25° C. Eggs 9 : 50 A.M. Sperm fresh. $AuCl_3$ 1 : 750,000.

1 : 750,000 was used in this experiment. Such a concentration has a p.H. of 8.0 and has no visible effect on the spermatozoa.

2. *Copper Chloride.*—Lillie ('21) investigated the effect of

copper salts of the fertilization reaction of *Arbacia* and found that there was a membrane-inhibiting point at 1 : 500,000 He also determined the cleavage-toxic concentration as 1 : 62,500. A repetition of this work confirmed the determinations that he made save that for one of the females employed it was found necessary to use a concentration of 1 : 37,500 $CuCl_2$ to completely inhibit cleavage. As has been stated above, the cleavage-toxic concentration varies somewhat, both for the eggs of different females and for the different physiological conditions existing in the eggs of the same female.

3. *Zinc Chloride.*—Zinc, it will be recalled, is one of the metals which formed such a precipitate that a cleavage-toxic concentration could not be determined. In the weaker solution necessary to completely inhibit the formation of membranes, however, the salt could be used with little difficulty. The usual series of experiments for the determination of this point was set up. The result of such an experiment may be found in Table II*a*.

TABLE II*a*.

Concentration of $ZnCl_2$ in Sea-water.	Per Cent. Membranes.	Per Cent. 2 Cell.	Per Cent. 4 Cell.
1 : 250,000	96	76	Present.
1 : 200,000	6	5	"
1 : 175,000	0	0	0
1 : 150,000	0	0	0
1 : 125,000	0	0	0
Inseminated control in sea-water	96	96	96
Uninseminated control in sea-water	0	0	0

Test of membrane-inhibition of $ZnCl_2$. July 5, '22. ♀ *C*. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. Exp. 2 : 30 P.M. Insemination immediate. All membranes narrow.

Table II*b* is a record of the viability of eggs which were inseminated in normal sea-water and transferred to the $ZnCl_2$ solution at intervals as indicated. It will be noted that when good membranes are raised, the toxicity of the membrane-inhibiting concentration on cleavage is not very marked. When narrow membranes have been raised the viability is much poorer.

4. *Lanthanum Chloride.*—Gray ('15) found that cerous chloride in a very weak concentration, would aggregate the sperm of *Arbacia*. He also found that by the addition of a small amount

TABLE II*b*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.	Per Cent. 2 Cell.	Remarks.
Immediate	0	0	
30 sec.	95	63	33 per cent. narrow membranes.
1 min.	100	82	Memb. good.
2 "	100	98	" "
2 "	100	98	" "
4 "	100	100	" "
8 "	100	100	" "
15 "	100	100	" "
30 "	100	100	" "
Inseminated control in sea-water	100	100	

Viability test. 1 : 150,000 $ZnCl_2$. July 5, 1922. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. ♀ *A*. Exp. 2 : 45 P.M. Temp. 20° C.

of NaOH, the sperm could be reactivated. Because of this, I made a few additional experiments on the sperm of *Arbacia* with $LaCl_3$ solutions. In the case of the immediate insemination in the lanthanum chloride solution, I found that, at a concentration of *N*/4,090 or 1 : 50,000, membranes are not raised. I then made up a thick sperm suspension by adding a number of drops of dry sperm to 7.5 c.c. of the $LaCl_3$ solution and observed that the sperm clumped and that their activity was much inhibited. The sperm in this solution were, however, reactivated by the addition of a drop of *N*/500 NaOH. I then made a similar suspension and added one drop of concentrated egg sea-water. The sperm immediately became very active and gave a very definite agglutination reaction.

After the membrane-inhibitory concentration had been established as 1 : 50,000, this solution was made up replacing the normal sea-water with concentrated egg sea-water, *i.e.*, egg sea-water of high agglutinating power. When eggs were inseminated in such a solution, the percentage of membrane formation rose from 0 per cent. to 64 per cent. to 72 per cent. It may thus be seen that egg sea-water has a great protective action in lanthanum-inhibition.

The viability test of the normally inseminated eggs in 1 : 50,000 $LaCl_3$ solution showed that this concentration is very slightly toxic to cleavage. As will be recalled, a cleavage-toxic concentration could not be determined because of the great amount of precipitation.

5. *Aluminium Chloride.*—The tests with $AlCl_3$ were much like those for the metals already described. The results were also much the same. It was found that a concentration of 1 : 40,000 (p.H. 7.2) would completely inhibit membrane elevation and that a concentration of 1 : 10,000 (p.H. of less than 6.6) was toxic to cleavage when the eggs were transferred to it after insemination in normal sea-water. As in the case of $LaCl_3$, the sperm were clumped when added to the solution, but when NaOH or egg sea-water was added, this clumping was corrected and the sperm became active. When the egg sea-water was added, the sperm agglutinated. Table IV. shows the result of

TABLE IV.

Time of Transfer after Insemination in Normal Sea-Water.	Per Cent. Membranes.	Per Cent. 2 Cell.
Immediate	0	0
30 sec.	80	20
1 min.	97	80
2 "	97	92
4 "	97	94
8 "	97	97
15 "	97	96
30 "	97	97
Inseminated control in sea-water	97	98
Uninseminated controls.		
a. Sea-water	0	0
b. $AlCl_3$	Eggs clumped.	

Viability test in 1 : 40,000 $AlCl_3$. June 19, 1922. ♀ *A.* Eggs and sperm fresh. Insemination 1 : 25 : 7.5. Exp. 1 : 00 P.M. Temp. 21.5 C.

a viability test on eggs inseminated in normal sea-water and later transferred to the membrane-inhibiting concentration of the salt. It will be noticed by an examination of this table, that the membrane-inhibiting concentration is more toxic to the first cleavage than is the case with the other metallic salts.

A test of the increase of membrane elevation due to an increase of sperm concentration showed that the percentage was raised from 0 per cent. to 32 per cent. on increasing the amount of sperm from 1 drop to 15 drops of the 1 : 25 suspension in immediate inseminations.

6. *Platinic Chloride.*—In the case of no other metallic salt studied is there as sharp a line of demarkation between concentrations which permit of membrane elevation and those which are absolutely inhibitory, as with $PtCl_4$. A stock solution was

made from the crystals which was .9 per cent. in strength and all the concentrations were made up by adding this to sea-water. The test for the membrane-inhibitory concentration shows that at 1 : 16,666, there is membrane elevation to equal the control, while at 1 : 11,111, there is a complete inhibition of membrane elevation. The p.H. of the latter concentration is 7.4. In this case each egg may be surrounded by from twelve to sixteen very active sperm. They strike the membrane and remain agglutinated to it though they are unable to incite any cortical change whatever. The agglutination of the sperm, by a drop of egg sea-water, in such a $PtCl_4$ solution when the suspension is made up directly in the solution, is good. The inhibition comes after the agglutination and before the cortical response of the egg can show itself, *i.e.*, it occurs in the latent period.

Not only is there a sharp distinction between concentrations which allow complete membrane elevation and those which are absolutely inhibitory, in the case of this metal, but the membrane-inhibiting concentration and the cleavage-toxic concentration are both more rapidly toxic to the eggs than is the case with the other metals studied. Table V*a* shows the results of a viability

TABLE V*a*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage
Immediate	0	0
30 sec.	88	44
1 "	92	50
2 "	96	62
4 "	98	84
8 "	98	84
15 "	98	98
30 "	98	97
Inseminated control in sea-water	98	98
Uninseminated control in sea-water	0	0

Viability test in 1 : 11,111 $PtCl_4$. June 30, 1922. Eggs 9 : 00 A.M. ♀ *A*. Sperm fresh. Insemination 1 : 25 : 7.5. Experiment 10 : 00 A.M. Temp. 21° C.

test at the membrane-inhibiting concentration of this salt; Table V*b* shows the effect of such a solution on eggs which have been forced to produce membranes by the use of an excess of sperm; Table V*c* shows the result of a viability test at the cleavage-toxic concentration. All of these tests show the great poisonous effect of the solution on the egg. The test with the

TABLE V*b*.

Drops of 1 : 25 Sperm Suspension.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1	0	0
2	0	0
4	2 narrow.	0
8	70 "	0.5 irregular.
12	80 "	0
16	100 "	1
Inseminated control in sea-water	100 good.	100
Uninseminated control in sea-water	0	0

Effect of increased sperm concentration in immediate insemination in 1 : 11,111 $PtCl_4$. June 30, 1922. Eggs as in Table V*a*. Exp. 10 : 00 A.M. Temp. 21° C.

increased sperm concentration shows the resistance of this solution to enforced insemination as well. Only after the eggs have been left in normal sea-water for four minutes after insemination therein, does the viability in Table V*a* begin to approach the normal control. At this same concentration, 1 : 11,111, while 100 per cent. membrane elevation may be produced by increase of the sperm concentration from one to sixteen drops of the 1 : 25 suspension, only 1 per cent. of the eggs cleave and these are irregular.

TABLE V*c*.

Time of Transfer after Insemination in Normal Sea-water.	Per Cent. 2 Cell.	Per Cent. 4 Cell.
10 min.	0	0
20 "	0	0
30 "	0	0
45 "	2	0
Inseminated control in sea-water	96	96

Viability test in 1 : 1,945 $PtCl_4$. July 1, 1922. Eggs 2 : 00 P.M. Sperm fresh. Insemination 1 : 25 : 7.5. Membranes 100 per cent. Experiment 3 : 30 P.M. Temp. 22° C.

A test of the cleavage-toxic point gave that determination as 1 : 1945. As soon as the eggs were transferred to such a solution after insemination in normal sea-water, they become crenate. Such a solution allowed no cleavage up to 45 minutes when the eggs were transferred to it at intervals after insemination in normal sea-water. The results of this experiment are given in Table V*c*. Cleavage took place in 48 minutes in the controls.

7. *Lead Chloride.*—$PbCl_2$, when added to sea-water precipitates very freely. This precipitate is not so heavy, however, that the membrane-inhibiting concentration cannot be determined as 1 : 4,000 or *N*/554 (p.H. 7.0.). Some precipitate was present

even at this concentration so that the amount of the salt really in solution could not be figured. Sperm will agglutinate readily in such a solution, though the eggs become clumped.

8. *Nickel Chloride.*—A series of tests, made with $NiCl_2$, follow so closely the general behavior of the metallic salts already described that but little discussion is necessary here. It was found the membrane inhibition first becomes evident at about 1 : 30,000 when the percentage of membranes raised fell from 100 per cent. to 80 per cent. in immediate inseminations, while at 1 : 5,000, $N/324$, membrane elevation is completely supressed. Such a solution, when used in a viability test, shows a decided effect on the production of cleavages other than the first. The effect is shown not only by the percentage of cleavage, but also in its regularity.

When tests were made to establish cleavage-toxicity, the concentration required was found to be 1 : 600 or $N/39$ −. Agglutination of the sperm is good in 1 : 5,000 $NiCl_2$ when one drop of the egg sea-water is added to 7.5 c.c. of thick sperm suspension made up in the membrane-inhibiting concentration. The sperm are active in such a solution even before the addition of the egg sea-water.

9. *Cadmium Chloride.*—Cadmium differs from the other metals studied in only one respect and there the difference is quantitative and not qualitative. When eggs are placed in the membrane-inhibiting concentration, which is 1 : 1,333 + or $N/122.1$, and after various exposure times, are transferred to normal sea-water and inseminated, there is a very marked poison effect after two minutes exposure. Not only does two minutes exposure cause very irregular development, even at the first cleavage, but it tends to inhibit the elevation of membranes. After four minutes, there were 98 per cent. membranes formed and 12 per cent. were narrow. After one hour, all were narrow. When eggs were exposed to the solution for one hour and then insemiated, the first cleavage gave 16 per cent. regular forms and 42 per cent. irregular.

The effect of this concentration on the sperm is very marked. They agglutinate readily with egg sea-water and can be activated by one drop of $N/500$ NaOH or egg sea-water though they are

quiescent in the solution alone. The percentage of membranes was raised from 0 per cent. to 84 per cent. by increasing the sperm concentration at insemination from one drop of 1 : 25 to 12 drops of the same suspension. The higher sperm concentration of this test resulted in much polyspermy.

It would be expected that by increasing the concentration of the $CdCl_2$ in solution, a cleavage-toxic point could be determined. I was successful in this; such a concentration is 1 : 250, or N 23 —.

10. *Cobalt Chloride.*—When a series of tests was made to establish the concentration of $CoCl_2$ necessary to inhibit the elevation of membranes, and the concentration necessary to inhibit completely the formation of the first cleavage in eggs which have been inseminated in normal sea-water and transferred to the solution five minutes after the insemination, it was found that both are much higher that in the other metals w th which I worked. The membrane-inhibitory point was found to be 1 : 1373, or N/89.245, while cleavage-toxicity occurred at 1 : 100 or N/6.5. As was the case with Cadmium, though not to such an extent, the toxicity of even the membrane-inhibiting concentration on eggs which had been inseminated in normal sea-water and transferred to it, was very marked.

Agglutination is good in the solution. The membrane-inhibiting concentration makes the sperm sluggish but this harmful effect is corrected by the addition, of the NaOH or a drop of the concentrated egg sea-water.

11. *Mercuric Chloride.*—As has already been stated above, there is one metallic salt which is irregular in its behavior. This is Mercuric Chloride. All the other salts with which it has been possible to work have a double effect on the eggs of *Arbacia*. The first effect is at the weaker concentration and is on the phenomenon of membrane elevation; the second effect is constantly at a greater concentration and is dependent on the time of exposure of the gametes for the intensity of its action. It modifies materially the viability of the eggs and I have termed it "cleavage-toxicity." The tests made with $HgCl_2$ show that there was no concentration which would inhibit the elevation of membranes and allow cleavage if eggs were placed in it five minutes after they were inseminated in normal sea-water.

TABLE VI*a*.

Concentration of $HgCl_2$ in Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1 : 1,000,000	100	16
1 : 800,000	100	12
1 : 600,000	100	0
1 : 400,000	100	0
1 : 300,000	100	0
1 : 200,000	100	0
1 : 100,000	52	0
1 : 75,000	48	0
Inseminated control in sea-water	100	100
Uninseminated control in sea-water	0	0

Test with $HgCl_2$. July 11, 1922. 11 : 20 A.M. Eggs and sperm fresh. Insemination 1 : 25 : 7.5. Eggs ♀ *C*. Temp. 20° C. Concentrations greater than 1 : 300,000 showed cytolysis in 20 minutes.

For example, an examination of Table VI*a* will show that at 1 : 600,000, $HgCl_2$ is toxic to cleavage, while there is 100 per cent. elevation of membranes which are wide and to all appearances absolutely normal. In those concentrations in which cleavage does not follow membrane elevation, cytolysis commences within an hour for those eggs with membranes. Eggs without membranes are more resistant. It appears from Table VI*b* that at 1 : 7,500 $HgCl_2$, membranes are not raised. At first glance it would be thought that this is a direct inversion of the ratio found to hold for the other metals where membrane-inhibition occurs at a lower concentration than cleavage-toxicity. An examination of the effect of such a solution on the sperm shows that they are instantly paralyzed by it and that this paralysis is not reversible, *i.e.*, neither NaOH nor concentrated egg sea-water will activate the sperm in it. This immediately differentiates such membrane-inhibition from that described for the other metals.

Lillie ('21) notes that the action of $HgCl_2$ is very different from that of $CuCl_2$. He notes that "the initial stages are relatively little affected, . . . the susceptibility increases as fertilization progresses" (page 140). He also notes that "mercury also suppresses the movements of the spermatozoa at great dilution." According to his data (Table VII.), a concentration of 1 : 625,000 completely inhibits cleavage while 1 : 15,625 prevents membrane elevation. He states further, that at the latter concentration, sperm are "paralyzed instantly." My observations are in accord with the above, save that it was found

necessary to use a concentration of 1 : 7,500 $HgCl_2$ to instantly paralyze sperm and consequently to completely inhibit membrane elevation (Table VI*b*). Lillie considered that the action of

TABLE VI*b*.

Concentration of $HgCl_2$ in Sea-water.	Per Cent. Membranes.	Per Cent. 1st Cleavage.
1 : 75,000	86	0
1 : 50,000	70	0
1 : 25,000	40	0
1 : 20,000	30	0
1 : 15,000	20	0
1 : 10,000	4	0
1 : 7,500	0	0
Inseminated control in sea-water	98	99
Uninseminated control in sea-water	0	0

Test of $HgCl_2$. July 21, 1922. 3 : 04 P.M. Temp. 26° C. Eggs ♀ *C*. Eggs and sperm fresh. Insem. 1 : 25 : 7.5.

mercury on membrane-inhibition was like that of copper but at a much higher concentration. I would prefer to consider that the action of $HgCl_2$ is a poisoning of the sperm, and not the type of inhibition that is present in copper-inhibition.

In most of the metallic salt solutions, the effect is to make the membranes narrow as the concentrations approach the strength necessary for membrane-inhibition; $HgCl_2$ has exactly the opposite effect. When eggs are inseminated in solutions which do not paralyze the sperm, membranes elevate immediately and such membranes are extremely wide. The solutions alone will produce membranes on eggs in concentrations greater than 1 : 150,000 as has also been observed by Lillie ('21). These membranes form much later than those produced by insemination so that it is easy to differentiate between the two. Eggs with membranes cytolyze very rapidly when left in $HgCl_2$ solution.

It would thus appear that, in comparing the action of the Mercuric salt with those of the metals already described, the only great difference is in the failure of the $HgCl_2$ to show any concentration which prevents the action between viable egg and sperm and consequently the initial response of the cortex of the egg.

b. Comparative Tests.

The preceding part is devoted to individual tests on the behavior of various salts. These tests were made with separate

lots of eggs, under different conditions, and are therefore not as suitable for comparison as they would be had they all been done simultaneously with one set of eggs. Three sets of experiments were therefore set up for the purpose of comparison.

The experiment which appears in Table VII*a*, was made to

TABLE VII*a*.

Time of Transfer after Insemination.	Percentage First Cleavage. Chlorides of:									
	Au[1]	Cu[1]	Zn	La	Al	Pt	Pb	Ni	Cd	Co
Immediate	0	0	0	0	0	0	0	0	0	0
30 sec.	29	38	87	98	94	96	80	70	80	80
1 min.	46	64	96	98	92	95	84	78	87	92
2 "	80	89	98	100	97	96	92	90	98	94
4 "	84	98	100	100	100	98	98	92	100	98
8 "	84	99	100	100	100	99	100	98	100	99
15 "	92	99	100	100	100	100	100	100	100	99
30 "	96	99	100	100	100	100	100	100	100	99
Inseminated control in sea-water	98	99	100							

Comparative test with membrane inhibitory concentrations. July 15, 1922 Experiment 10 : 15 A.M. Temperature 21° C. Sperm and eggs fresh. Insemination 1 : 25 : 7.5. Membranes formed well, save in immediate inseminations.

[1]In the above experiment, the results for Au and Cu are interpolated.

show the effect of the various solutions on the viability of the same lot of eggs under the same conditions. In each case the concentration used is that of membrane-inhibition. In this table it will be noted that only the figures for the first cleavage are given. In all save the immediate inseminations, membranes were present on a high percentage of the eggs. The table, therefore, emphasizes the great difference between the inhibition of initial and subsequent events, and shows the great similarity of the action of all of the salts. In this table, the figures for $AuCl_3$ and $CuCl_2$ are interpolated. It was found, however, that both of these membrane-inhibiting concentrations proved more toxic to cleavage than those of the other salts. I have not used $HgCl_2$ in this test as it shows no membrane-inhibiting point.

The experiment summarized in Table VII*b* was performed to determine the time when superficial cytolysis first begins to appear in uninseminated eggs placed in membrane-inhibiting

concentrations of each of the metallic salts. Readings were made at the end of fifteen minutes, one half hour, and at half-hour intervals thereafter for four and one-half hours. In this

TABLE VII*b*.

Metallic Salt.	Superficial Cytolysis Begins. Hours.
$AuCl_3$	$\frac{1}{4}$
$HgCl_2$	$\frac{1}{2}$
$CuCl_2$	1
$ZnCl_2$	$1\frac{1}{2}$
$LaCl_3$	2
$AlCl_3$	2
$PtCl_4$	$3\frac{1}{2}$
$PbCl_2$	$3\frac{1}{2}$
$NiCl_2$	$3\frac{1}{2}$
$CdCl_2$	$3\frac{1}{2}$
$CoCl_2$	4
Control in sea-water	None in $4\frac{1}{2}$ hours.

Test of time of cytolysis in membrane-inhibitory concentrations. Eggs, 1 drop to 7.5 c.c. solution. 10 : 45 A.M. 7/26/22. Eggs fresh, 2 ♀s. Temp. 22.5° C.

experiment, one drop of the uninseminated eggs was placed in 7.5 c.c. of the membrane-inhibiting concentration of each of the metallic salts. The container, which was a Syracuse watch-glass was then rotated to insure a thorough mixing of the gametes and the solution.

TABLE VII*c*.

Metallic Salt.	Action Alone.	1 Drop *N*/500 NaOH Added.	1 Drop Concentrated Egg Sea-water Added.
$AuCl_3$ ..	Active.	Very active.	Very active. Agglut.
$CuCl_2$ ..	"		" " "
$ZnCl_2$ ..	"	Paralyzed.	" " "
$LaCl_3$ ..	Inactive. Clumped.	Clumped. Some activity.	" " "
$AlCl_3$ ..	Active. Clumped.	Clumped. Very active.	" " "
$PtCl_4$ ..	Active.	Very active.	" " "
$PbCl_2$ ..	Active. Some clumping.	" "	" " "
$NiCl_2$ ..	Active.	" "	" " "
$CdCl_2$ ..	Quiescent.	" "	" " "
$CoCl_2$ ..	Sluggish.	Fairly active.	" " "
$HgCl_2$ ..	Paralyzed.	No change.	No change.

Effect of membrane-inhibitory concentrations on the sperm. July 18, '22. Sperm suspension made with dry sperm in 7.5 c.c. of the solution. Sperm from two males.

The experiment in VII*c* was with the membrane-inhibiting concentration of each of the metals and its effect on the sperm.

In this series, a sperm suspension was made by adding a number of drops of the dry sperm to 7.5 c.c. of the membrane-inhibitory concentration of the metallic salt, and the effect of the solution on the behavior of the sperm was noted. A drop of NaOH ($N/500$) was then added to one preparation and its effect noted. To another preparation, a drop of concentrated egg sea-water was added. The results may be seen in Tab'e VII*c*.

c. Summary.

In summarizing the results of the foregoing experiments, it may be generally stated, that, with the exception of $HgCl_2$, all of the metals studied have three effects on the fertilization period of *Arbacia*. The first and second occur at a greater dilution than the third and involve membrane elevation. When eggs are inseminated directly in certain solutions, they give no cortical response, *i.e.*, the effect is immediate and prevents any reaction between the gametes themselves; if, however, eggs are placed in this same solution after cortical discharge has begun but before it is complete, a "narrow membrane" results which may be attributed to an incomplete cortical response. The third involves the subsequent events and requires a higher concentration of the metallic salt. It seems from the data to be cumulative in its action. When the eggs are placed in the solutions five minutes after the insemination in normal sea-water, the time factor amounts to approximately forty minutes, as first cleavage is used as an indicator. If the eggs are transferred at a later time, first cleavage may be only partially inhibited but there will be no second cleavage. That is to say, the time required for the salt solution to produce its toxic effect is, in these experiments, about thirty-five or forty minutes. Table VIII gives a comparison of the concentrations necessary to produce these effects. The order of toxicity of the metallic salts will be seen to be the same for both membrane effects as well as subsequent events. The general results are in accord with those noted by Lillie in his work on copper. Mercury, as has been observed above, is an exception to this general rule.

In each case, as can be seen by reëxamination of Table VII*c*, sperm may be caused to agglutinate in the membrane-inhibitory

TABLE VIII.

Metallic Salt.	Membrane-inhibition.		Cleavage-toxicity.	
	Parts.	Normality.	Parts.	Normality.
$AuCl_3$.....	1 : 1,500,000	*N*/151,800	1 : 375,000	*N*/37,912.5
$CuCl_2$.....	1 : 500,000	*N*/ 42,030	1 : 62,500	*N*/ 8,405
$ZnCl_2$.....	1 : 150,000	*N*/ 10,215		
$LaCl_3$.....	1 : 50,000	*N*/ 4,090		
$AlCl_3$.....	1 : 40,000	*N*/ 1,780	1 : 10,000	*N*/ 445
$PtCl_4$.....	1 : 11,111	*N*/ 936	1 : 1,945	*N*/ 163.87 —
$PbCl_2$.....	1 : 4,000	*N*/ 554		
$NiCl_2$.....	1 : 5,000	*N*/ 324	1 : 600	*N*/ 39 —
$CdCl_2$.....	1 : 1,333.33	*N*/ 122.1	1 : 250	*N*/ 23 —
$CoCl_2$.....	1 : 1,373	*N*/ 89.245	1 : 100	*N*/ 6.5
$HgCl_2$.....			1 : 600,000	*N*/81,000

The above table shows the series of metals as established for both membrane and cleavage inhibition in *Arbacia*. With the exception of $HgCl_2$ they are in the order of increasing concentration.

concentrations by the addition of egg sea-water, thus demonstrating that the action is not primarily on the sperm before their interaction with the female gametes.

V. DISCUSSION.

There are three periods definable in *Arbacia* from the moment of insemination up to the first cleavage. The first involves the behavior of the spermatozoön alone and includes its activation, migration to the egg, and its agglutination to the egg. The second involves the reaction of both egg and sperm, and lasts from the end of the latent period to the completion of the cortical activation of the egg. It can, of course, be subdivided. The third is concerned with the rotation of the sperm head within the egg cytoplasm, and the migration of the egg nucleus. In other words, it is the preparation for the union of the germ nuclei and the preparation for cleavage. A latent period comes between the time of the agglutination of the sperm to the egg and the initial events of cortical activation, as described by Lillie ('21). It belongs to the first period described rather than to the second.

The differentiation between the first and second periods is very markedly emphasized by the foregoing experiments. When eggs are placed in solutions indicated in the text as membrane-inhibitory, and immediately inseminated, the sperm are seen to

agglutinate to the egg. This inhibition, therefore, very evidently occurs between the periods of agglutination and of activation, or during the latent period. It may be said that the egg is held at the latent period by the solution. As soon as the agglutination is seen to occur, there is a block interposed, and the reaction ceases. At the time when this block is first interposed, there is no great harm done to the eggs. If they are removed to sea-water and inseminated, the sperm penetrate and cortical activation ensues. This seems to show that the action involved affects only the very external parts of the gametes and is easily removed. After an exposure of slightly longer duration, the viability of the eggs becomes poorer, but this I would consider to be due to the penetration of the salt into both the cortex, and, later, the central part of the egg and consequently, to a very different mechanism from that which prevents the elevation of the membrane.

There are a number of physical and chemical phenomena which may be responsible for this inhibition of membrane formation. The theory of chemical combination was suggested by Lillie. He remarks ('21) the similarity of the effective concentrations found to hold in the "cleavage toxicity" of his work and in the enzyme poisoning discussed below. The physical action may be of two kinds. It may be an adsorption phenomenon resulting in either a change of the electrical charge on the membrane which is present before fertilization or an adsorption by molecular groups. Heesch ('21) finds that the charge on cell membranes of *Lycopodium* spores, leucocytes, and yeast cells can be reversed by the use of $La(NO_3)_3$ solutions. Whether this action is the same as that in membrane-inhibition or not, is an open question. Membrane-inhibition might be due to a change of charge of the egg membrane, or to an adsorption of the metallic ions by some of the complex molecular groups in the egg protoplasm. Were these groups those of the activable substance, this adsorption would explain the partial activation described above. The first of these would involve an electrical action while the latter would result in a chemical inactivation though not a chemical reaction.

Lillie ('21) noted the close similarity between the concentra-

tions of $CuCl_2$ required for cleavage-toxicity and that found by v. Euler and Swanberg ('20) to be toxic to the action of saccharase. McGuigan ('04), in an extensive set of tests of the effect of the metallic salt solutions on the action of diastase, determined the concentrations which inhibit its action for a series of metals. Olsson ('21) also worked on the effects of the various salts of silver and copper on the action of amylase. v. Euler and Swanberg ('21—II) found that the action on saccharase by organic substances was not effected by the temperature. They conclude that there is a binding of the enzyme through the aldehyde group. McGuigan compares the action of the salts to the solution tensions of their ions, though he finds certain irregularities in the series. His results for diastase and those of v. Euler and Swanberg for saccharase are in very close agreement.

The data concerning membrane-inhibition in *Arbacia* do not agree with the results noted by the above authors for enzyme-poisoning. While chemical activity could explain the inhibition effect satisfactorily, it would be difficult to conceive an immediate correction of this, effect if the inhibiting factor were removed. As has already been noted, when the eggs are removed to sea-water from a solution in which inhibition is complete, if the exposure has not been too long, there is an immediate recovery and the eggs will produce membranes on insemination in the normal manner.

The present paper emphasizes the distinction to be made between the initial events in fertilization as they are indicated by the cortical activation, and the subsequent events which lead to the period of cleavage.

VI. Conclusions.

1. The chlorides of the following heavy metals inhibit membrane formation in *Arbacia* in the following order and concentrations;—Au—*N*/151,800, Cu—*N*/42,030, Zn—*N*/10,215, La—*N*/4,090, Al—*N*/1,780, Pt—*N*/936, Pb—*N*/554, Ni—*N*/324, Cd—*N*/122.1, Co—*N*/89.2. The effect of these concentrations upon the cortical response of the egg is immediate. Eggs inseminated 1 : 25 : 7.5 in these solutions immediately become bombarded by many sperm, no one of which is able to activate the cortex of the egg at the above concentration.

2. In contrast to the above, but showing the same general order, are the concentrations of the same metallic salts which are toxic to cleavage. These salts, with their toxic concentrations are;—Au—*N*/75,825, Cu—*N*/8,405, Al—*N*/445, Pt—*N*/163.87, Ni—*N*/39 —, Cd—*N*/23 —, Co—*N*/6.5. There was so much precipitation in the case of Zn, La, and Pb, that the concentration of these salts, toxic to cleavage could not be determined.

Mercuric chloride proved toxic to cleavage at *N*/81,000, while seeming to favor membrane elevation. At a concentration inhibiting membrane elevation, sperm were paralyzed immediately.

These concentrations vary slightly for different batches of eggs and show the influence of a time factor, thus giving an interesting contrast to membrane-inhibition, the concentrations of which are more constant and immediate in their action. These concentrations are greater in every case except Hg, than the membrane-inhibiting concentration of the same metal. Cleavage toxicity is a progressive, or a cumulative action.

3. These solutions are not immediately harmful to the gametes, but prolonged exposure is injurious to the eggs as can be shown by two sets of experiments.

(*a*) When the female gametes are exposed to the solutions for various periods of time and are then inseminated in fresh sea-water, the viability always stands in inverse ratio to the length of exposure.

(*b*) When the inseminated eggs are transferred to the solutions at intervals after insemination in normal sea-water, the length of exposure to the solution bears an inverse ratio to the viability of the eggs.

It will be seen in both (*a*) and (*b*) that the time factor is the important one. The action here is again cumulative.

4. With the exception of mercuric chloride, any harmful effect of these solutions on the sperm is corrected by the addition of a drop of egg sea-water.

LITERATURE.

1. **von Dungern, E.**
'02 Neue Versuche zur Physiologie der Befruchtung. Zeitschrift für allgemeine Physiologie, Bd. I.

2. v. Euler und Swanberg.
'20 Ueber Giftwirkung bei Enzymreaction.
I. Inaktivierung der Saccharase durch Schwermetalle. Fermentforschung, III. Jahrgang, pp. 330–393.
II. Inactivierung der Saccharase durch organische Stoffe. Fermentforschung, IV. Jahrgang, pp. 29–63.
III. Ueber den Einfluss von Kupffersulfat auf die Autolyse der Hefe. Fermentforschung, IV. Pp. 90–96.
IV. Electrometrische Messungen ueber die Bindung des Silbers und des Kupffers an Saccharase und an andere organische Verbindungen. Fermentforschung, IV. Jahrgang, pp. 142–183.
'21 Ueber die Regeneration inactivierte Saccharase durch Dialyse. Zeitschrift für Physiologische Chemie. Bd. 114, page 137.

3. Fol, H.
'79 Researches sur la Fécondation et le commencement de l'Hénogénie chez divers animaux. Genéve Soc. Phys. mem., XXVI, pp. 89–397.

4. Gray, J.
'15 Note on the Relation of Spermatozoa to Electrolytes and its bearing on the Problem of Fertilization. Quart. Jour. Micro. Sci., N. S., LXI., pp. 119–126.

5. Heesch, K.
'21 Untersuchungen ueber die Umladbarkeit von Zellen, Zellbestandteilen, und Membranen. Arch. f. d. Gesamt. Phys. Bd. 190, pp. 198–212.

6. Just, E. E.
'19 The Fertilization Reaction in *Echinarachnius parma*. I. Cortical Response of the Egg to Insemination. BIOL. BULL., Vol. 36, p. 1.

7. Lillie, F. R.
'13 Studies of Fertilization. V. The Behavior of *Nereis* and *Arbacia* with Special Reference to Egg-Extractives. Journal of Exp. Zoöl., Vol. 14, pp. 515–574.
'14 Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia*. Journal of Exp. Zoöl., Vol. 16, pp. 523–590.
'19 Problems of Fertilization. xii + 278 pp. The University of Chicago Press.
'21 Studies of Fertilization X. The Effects of Copper Salts on the Fertilization Reaction in *Arbacia* and a Comparison of Mercury Effects. BIOL. BULL., Vol. XLI., pp. 125–143.

8. McGuigan, H.
'04 The Relation between the Decomposition Tension of Salts and their Antifermentation Properties. The American Journ. of Physiology, Vol. X., pp. 444–451.

9. Mathews, A. P.
'04 The Relation between Solution Tension, Atomic Volume, and the Physiological action of the Elements. The American Journ. of Physiology, Vol. X., No. 6.

10. Olsson, U.
'21 Ueber Vergiftung der Amylase durch Schwermetalle und organische Stoffe. Zeitsch. f. Physiol. Chem., Bd. 114, pp. 51–71.

THE BEHAVIOR OF CALCIUM PHOSPHATE AND CALCIUM CARBONATE (BONE SALTS) PRECIPITATED IN VARIOUS MEDIA, WITH APPLICATIONS TO BONE FORMATION.

JAMES CRAWFORD WATT,

ANATOMICAL LABORATORY, UNIVERSITY OF TORONTO.

WITH THIRTY-EIGHT FIGURES IN THREE PLATES.

CONTENTS.

	PAGE
Introduction	280
Part 1. Microscopic Study of Precipitation:	
Technique	282
Reaction in aqueous solutions	283
Precipitation of calcium phosphate	283
Precipitation of calcium carbonate	284
Precipitation in colloidal solutions	284
Precipitation of calcium phosphate	285
Precipitation of calcium carbonate	285
Influence of other agents on the precipitate	288
Influence of different ions	290
Precipitation in blood serum	291
Precipitation in hyaline cartilage extract	291
Influence of hydrogen ion concentration	292
Disintegration of spherules	295
Number of forms assumed by precipitate	297
Part 2. Examination of Bone:	
Technique and material	298
Results of examination of bone	300
Part 3. Discussion:	
Theories regarding deposit of calcium salts	301
Reversibility of calcium reaction	305
Summary	307

INTRODUCTION.

There are at present three main theories presented in explanation of the manner in which the matrix of bone becomes impregnated with the two inorganic salts, calcium phosphate and calcium carbonate. These views may be briefly summarized as follows:

1. That the salts are deposited in the matrix by a precipitation *in situ* from the interaction of soluble salts in the blood and the tissue.

2. That the salts are excreted, or secreted, either with the matrix, or into the matrix, by the bone cells.

3. That a complex combination salt known as calcium carbonophosphate, carried in solution in the blood, is thrown out of solution in the bony matrix by a change in the carbon dioxide content of the tissue, and after precipitation is finally converted into the two components, calcium carbonate and neutral calcium phosphate, in the exact proportions found in bone.

The weight of evidence is at present in favor of the third view, especially supported by the work of Barillé and of Wells, but it seems to me that fully conclusive proof that this is the process has not yet been brought forward.

The precipitation theory, however, offers a very simple and plausible explanation, and so it was determined to investigate the behavior of bone salts on precipitation and see if any light could be shed on the actual manner of impregnation of the bony matrix.

An exact knowledge of the way in which the bone salts are added would be a great aid, indeed, to our understanding of the growth of bone, repair of fractures, changes in rickets and osteomalacia, and other kindred conditions. It would also throw light on the formation of calcareous patches in scar tissue and in arteriosclerosis, for Wells has shown that calcification and ossification are quite similar processes, and the same two calcium salts are present in both cases in exactly the same proportions.

This research work, as finally carried out, involved the microscopic study of the reactions whereby calcium carbonate and calcium phosphate were precipitated, both in separate solutions and also in the same solution, first in various aqueous media and then in certain colloidal ones. This was followed by a thorough examination of the unchanged matrix of many different bones in the mouse, the frog, the guinea pig, the dog, and human fœtuses of various ages.

"For experimental investigation of the artificial process will

furnish the best clue to a precise and certain knowledge of the natural one, by showing more clearly how much is due to physical agency"—Rainey.

PART I. MICROSCOPIC STUDY OF PRECIPITATION.

TECHNIQUE.

For simple examination of a reaction, a glass slide measuring 38 x 75 mm. was taken and on it were outlined two squares with sides of 20 mm. painted in melted paraffin, so that the wax formed the sides of a shallow cell. Into a cell were placed a small amount of the two reacting solutions to give the desired precipitate, and then a cover glass 22 mm. square was dropped on, supported by the wax cell wall. If it was desired to keep the specimen, melted wax was then painted all around the edges, and overlapping on to the cover glass. The wax was then coated with thick shellac, which in its turn overlapped slightly on to the glass. The cell was thus permanently sealed. Two cells could be made on one slide and the same reaction could be compared in distilled water and in a colloidal solution side by side.

It was found desirable to examine not only the precipitate formed immediately at the line of contact of the two reacting solutions, but also what was formed later, in more remote parts of the solution, during diffusion, and as the concentration of the dissolved salts became less and less with increasing precipitation. To do this required larger cells, and it was desirable to have a fairly uniform thickness of cell, which was accomplished as follows:

On a glass slide 38 x 75 mm. in size two thin parallel strips of mica dipped in melted paraffin were so placed as to support the long edges of a glass cover slip measuring 24 x 50 mm., which was placed upon them. These long edges were now thoroughly painted over with melted paraffin, which on hardening cemented the cover slip to the slide. A cell measuring from 0.1 mm. to 0.2 mm. deep was thus made, still open at both ends. By means of a fine glass pipette one of the reacting solutions, $M/10$ calcium chloride, was now introduced at one end until the cell was nearly two thirds filled. This end was then carefully dried and sealed with paraffin.

At the still open end was now introduced the other solution—for instance, $M/4$ sodium carbonate, or $M/4$ triple sodium phosphate—and the last border of the cell was then sealed and the slide labeled. All borders were then painted with thick shellac, which was allowed to slightly overlap the wax on to the glass.

To obtain a series of cells of uniform depth, a sheet of mica four inches by two inches in size was split until the required thickness was obtained, equal to that of a number two cover glass. It was cut across with scissors into two-inch strips about two millimeters wide. One sheet thus prepared will cut into approximately forty or fifty strips.

Cells filled with warm solutions sometimes showed broken cover glasses on cooling. Also in cells completely filled with solutions a slight amount of expansion due to change of temperature was sufficient to cause leakage by loosening the wax from the slide. And so to provide for permanency of the specimens all cells were so filled as to leave an air space to provide for contraction or expansion without undue strain, and were filled with solutions at room temperature, 20°–21° C.

Pure distilled water was used in all experiments, all solutions were filtered to make them perfectly clear, and chemically pure reagents were employed in every case.

Reaction in Aqueous Solution.

Precipitation of Calcium Phosphate.

Calcium phosphate was obtained by the interaction of $M/10$ solution of calcium chloride and $M/4$ solution of triple sodium phosphate in water. This strength of reagent gave plenty of precipitate, without it being so dense as to interfere with proper microscopic examination. As the two solutions were of different concentration, there was a different rate of diffusion on the two sides of the line of contact, with corresponding differences in rate and amount of precipitation.

Immediately that contact occurs between the two solutions a milky cloud appears, which grows rapidly in size. For a moment, although the cloud is visible to the naked eye, the solution seen microscopically is still clear, but an instant later it becomes vio-

lently agitated, and the agitation becomes quickly defined as being caused by very minute particles, which are in very rapid motion, both oscillatory and translatory. These particles, appearing only as small dots even under the highest magnification, grow in size, losing their motion as they increase in bulk, until finally they form the fine amorphous granules which gather in clumps, and masses, and fine films, to form the precipitate.

Precipitation of Calcium Carbonate.

Calcium carbonate was precipitated by the interaction of a $M/10$ solution of calcium chloride with a $M/4$ solution of sodium carbonate. The process here resembles that described above for calcium phosphate in the form of myriads of fine particles in rapid Brownian movement. All movement finally ceases as these particles agglutinate into large clumps, and then a remarkable change occurs, for the granular masses just formed fade from the sight, dissolving again into the solution, while scattered here and there larger, rapidly growing particles occur, which become defined as crystals, angular in outline, or as small spherical bodies, known as spherules, which are later converted into crystals as they increase in size (Figs. 1, 2, 3, and 25).

Two or three hours after the formation the precipitate is all crystalline. It is very rare to see a spherule persisting in pure aqueous solutions.

The appearance of precipitates of calcium carbonate in the form of spherules has been frequently noted before. As long ago as 1839 it was described by Link, who, in accordance with this phenomenon, thought that crystals at their first origin were fluid and only later became hard and angular, as solidification occurred.

PRECIPITATION IN COLLOIDAL SOLUTIONS.

The colloids employed were gelatin and egg albumen.

Various concentrations of gelatin were experimented with, ranging from an 8 per cent. solution, which solidified quickly, to a 1 per cent. solution, which remained fluid for some time and then formed a soft jelly. As the various concentrations gave similar results the weaker ones were used as a matter of routine, being easier to handle.

For albuminous solutions Merck's powdered soluble egg albumen was employed, water being added to make the approximate normal proportions given by Lillie for fresh albumen in the hen's egg, namely, albumen 12 per cent., water 88 per cent. by weight.

In 1857 Rainey published the first of a series of papers on the precipitation of carbonate and phosphate of calcium in colloids, using gelatin, albumen, and gum arabic. His best results were obtained with gum arabic, and he pointed out that the solutions of the gum holding the two salts reacting to give the precipitate should be of very different densities, so that diffusion and the consequent reaction would occur slowly and gradually. I have found, however, with albumen and gelatin, that if the reacting salts differ sufficiently in concentration, the concentration of the colloidal solution need not differ in order to secure good reactions. And so in many series of experiments 1 per cent. gelatin was used throughout, although in others this concentration was used only with calcium chloride, and a 4 per cent. solution with sodium carbonate or phosphate. Egg albumen was used always in the same concentration.

Precipitation of Calcium Phosphate in Colloids.

The employment of a colloidal solution seems to have little or no influence on the precipitation of calcium phosphate. The deposit is quite similar to that in aqueous solutions, being fine, granular, and amorphous (Fig. 36), and aggregating to form thin gelatinous veils and clouds. Results in egg albumen were similar to those in gelatin. These findings are in agreement with the work of Rainey, Harting, Biedermann, and others.

Precipitation of Calcium Carbonate in Colloids.

The reaction here is very characteristic. Calcium carbonate formed in the presence of colloids has a tendency to separate out and persist in the form of the spherules mentioned previously, which have been named calcospherites.

The first effect of the colloid is to very much emphasize and prolong the stage in which the particles are small and show active Brownian movement, apparently by preventing their early fusion into larger masses. In a short time, however, larger particles are

seen which grow partly at the expense of the smaller ones, which redissolve as described for aqueous solutions. But there are many small particles which apparently do not dissolve, but pass on directly over to the larger form of deposit, by forming a nucleus for the laying down of additional material from the clear solution.

The precipitate exhibits two separate forms. One of these is distinctly crystalline (Figs. 8, 10, 12, 13) and is found most densely at the primary line of contact of the reacting solutions where deposit was very rapid. Even here the colloid shows its influence, for the crystals are not perfect, but are deformed in various ways, and exhibit rounded angles and suppression of their typical shape.

The second form of deposit is in the shape of very perfect small spheres (Figs. 7–12, 29, 30, 31), which vary greatly in their transparency, markings, and size according to the solution in which they are formed.

Rainey obtained spherules in gum arabic only after the lapse of an hour or more. At first a faint nebulosity was seen, lasting in thin solutions of gum for one hour, in thick solutions for over a week, after which spherules occurred. In my work I have been able to identify these bodies in less than ten minutes, and in some solutions to see them attain a size of 30 μ in one hour. They grow rapidly and attain practically their full size in forty-eight hours, ranging then according to the solution in which they lie, from a size of 1 μ up to 120 μ. In Rainey's case they grew gradually for several months and ranged in size from 2 μ to 200 μ. I had one single example of a pear-shaped body which measured 120 μ x 300 μ.

Evidently gum arabic exhibits a more powerful colloidal influence on the precipitate, but gelatin or albumen will give excellent results in a very much shorter time, which is a valuable consideration.

Brownian movement in parts of some slides is still active after the lapse of a year, due to the colloid retaining some of the precipitate in its original, extremely small, granular form, preventing coalescence to form the larger particles seen in other areas where precipitation was denser and more rapid.

The process of coalescence of the small particles can be observed in favorable cases. Very small spherules showing active move-

ment travel about in the solution, colliding with and working past others. Suddenly two of these, instead of passing, will be drawn together, as though pulled by a strong attractive force, and will merge completely in one large spherule, which in its turn may absorb small ones. At these moments of coalescence the spherules give the observer the impression of being fluid, flowing together like two drops of water, and lead one to believe that Link was correct in interpreting these small spherules as liquid crystals. As these bodies become larger they lose their movement, settle down, and become of glassy hardness. There is also a later growth by addition of material from the surrounding clear solution.

Richards and Archibald have shown that the particles of precipitates at their first appearance seem to have a rounded outline to the eye even under high magnification, but by photographs, however, they have demonstrated that these particles are real crystals of typical shape. This condition is true for some particles of calcium carbonate formed in aqueous solutions, but in colloids a great deal of the deposit is in the form of particles which are round, and persist in this shape as they grow. There are other particles also, which clearly are not round, and give the crystalline form of deposit which occurs simultaneously with the spherules.

The spherules formed in gelatin (Figs. 13 and 31) are very perfect, with a smooth surface, and clear, transparent, glassy appearance. Those formed in albumen (Figs. 26 and 29) often show a slightly irregular periphery and give indications of their internal structure. They appear to be built up of concentric layers, and often exhibit an amorphous center. Extending from this center to the periphery is also a faint, but quite distinct, radial striation in certain cases. These appearances have already been well described and illustrated by Rainey, Harting, and Biedermann.

It ought to be noted here that Harting has been erroneously credited by many writers with the discovery, in 1872, of the calcospherites formed in colloidal solutions. Rainey described and gave accurate illustrations of these bodies in 1857, and had observed them as early as 1849, and so deserves the credit of this discovery.

Combined precipitates of calcium carbonate and phosphate (Fig. 36) show each salt acting quite independently, the phosphate occur-

ring in its usual amorphous form, the carbonate in typical crystals and spherules. Similar conditions were found by Rainey in gum arabic.

The colloids gelatin and albumen were chosen as being artificial media easily prepared which might be looked on as approximately comparable respectively to the bony matrix and to the blood serum or to cellular tissues. Reactions in gelatin might give a clue to those in bone, and similarly reactions in albumen might indicate processes of calcification in other tissues. After studying the reactions in these simple media, they were further modified by the addition of various substances which are either normally or pathologically found in the blood or tissues, to see what effect these special substances would exert.

Further experiments were also carried out to study the effect of the hydrogen-ion concentration of the colloid. To complete this stage of the work reactions were followed in cartilage extract and in blood serum. Then specimens of bone from various animals were examined to see if there was any correlation between the structure of their matrix and the results obtained experimentally in the various media.

The Influence of Other Agents on the Precipitate.

The substance whose influence was to be investigated was added in equal concentration both to the sodium carbonate or phosphate, and to the calcium chloride solutions which were to react to form the precipitate. The new agent was thus balanced so that its action could not be one sided.

The agents used and their concentration in solution were:

M/10 sodium chloride, same proportion as in human blood serum.
0.1 per cent. dextrose, same proportion as in human blood serum (Gradwohl).
0.05 per cent. urea, same proportion as in human blood serum.
0.1 per cent. lecithin. Blood serum contains 0.7 per cent. (Wells).
1.0 per cent. acetone. Simulating conditions in acidosis.
0.5 per cent. ethyl alcohol.

The effects produced on the precipitation of calcium carbonate were quite definite for some of these substances, and in some in-

stances very remarkable changes of form resulted. The influence on calcium phosphate was much less striking, as this deposit remained in the same granular, amorphous form in all cases, and the results were mainly a slight difference in the average size of granules, and also differences in the speed and completeness of precipitation similar to those which will be noted in detail below for calcium carbonate, to which latter deposit alone the succeeding paragraphs relate.

Addition of Sodium Chloride.

The main effect noted on adding sodium chloride was the speeding up of the reaction. Brownian movement ceased sooner, crystallization was more rapid, and in most cases the main portion of the precipitate consisted of smaller crystals and spherules, and in the colloids the crystals were of very poor shape.

Addition of Dextrose.

The influence of this compound was slight and seemed to consist mostly in added transparency of the crystals and spherules. There was also slight modification in shape.

Addition of Urea.

Even in aqueous solutions some spherules were formed when urea was present. The effect in colloidal solution (Figs. 30 and 35) was to favor increase in size of the spherules, with a marked tendency toward fusion, so that many double forms and dumbbell-shaped bodies were seen. Urea thus shows an influence quite the opposite of sodium chloride upon the precipitate. It evidently favors growth of this deposit and suggests the idea that the presence of urea in blood and in urine may aid in the deposit of calcium salts in the calcareous incrustation of sclerotic arteries and the growth of renal and vesical calculi.

Addition of Lecithin.

Lecithin, even in small amounts, 0.1 gm. in 100 c.c. water gives a turbid solution even after filtering, but microscopically the solution appears clear, so that it is evidently a colloidal one.

The lecithin has a remarkable influence on the precipitation of

calcium carbonate. Even in aqueous solution (Figs. 4, 5, 6) spherules occur in great numbers, and many of these are of a large size not obtainable in other solutions. There is also a distinct tendency toward fusion of spherules in contact with each other, so that double and multiple forms are numerous. Beautiful rosettes (Fig. 5) were present, due to the adhesion of many oval-shaped bodies.

In gelatin and albumen (Figs. 10 and 11) the same phenomena occurred, producing magnificent rosettes and very large spherules measuring 120 μ in diameter.

Lecithin thus has a very typical colloidal action on the precipitate, favoring the spherical forms of deposit. This compound is found in blood in seven times the strength it was employed here, and is also found in bone and other tissues, so that its action on a precipitation of bone salts, if such occurs, ought to be appreciable. This point will be discussed later.

Addition of Acetone.

The presence of acetone (Fig. 12) hastens the process of precipitation, making it occur very rapidly, and also makes the initial deposit at the moment of contact much denser and more complete. This point is rather suggestive. It leads to the question as to whether or not conditions of acidosis such as accompany serious cases of nephritis and diabetes have an influence favoring the progress of calcification in the arteriosclerosis so often found in these cases.

Addition of Ethyl Alcohol.

The alcohol had no appreciable effect upon precipitation except to add to the optical clearness of some preparations.

THE INFLUENCE OF DIFFERENT IONS.

The calcium carbonate was precipitated in separate cells by the carbonates of sodium (Figs. 1 and 4), potassium (Figs. 2 and 5), and ammonium (Figs. 3 and 6), so that the solutions contained the three last-named ions as the only variable, all other factors being equal. In aqueous solution the crystal form of the calcium carbonate in the three cases was noticeably different. In colloidal

solutions the spherules seen were very different in their appearances in each set of slides.

The most noteworthy change was seen where the ammonium salt was used, the deposit being much coarser, with rougher, more irregular surfaces, and as a rule much greater in size and with more tendency toward fusion and toward clumping into masses.

The whole series for each ion, including those slides with the six accessory agents described above, showed similar tendencies to the plain, unmodified reaction. Each series consisted of 21 slides, consisting of 7 in water, 7 in gelatin, and 7 in albumen.

Precipitation in Blood Serum.

Clear serum was pipetted off from test tubes of dog's blood allowed to stand for some hours in a refrigerator. The precipitation of calcium phosphate in this serum showed the usual granular amorphous character. Calcium carbonate separated out almost entirely in the form of spherules, many of which were quite large. The process of precipitation in blood serum was very complete, covered practically the whole area of the slide, and seemed to be greater than usual in amount. This appearance suggested the thought that the sodium carbonate and phosphate of the blood aided the reaction, making precipitation greater in amount, but further consideration made this seem unlikely, as the usual amount of carbonate or phosphate was added to the blood, and this alone was sufficient to precipitate more than the total amount of calcium, without the aid of the salts naturally in the blood.

Mixtures of carbonate and phosphate present the usual character, each salt being precipitated in its own peculiar form and being thus easily identified (Fig. 36).

Precipitation in Hyaline Cartilage Extract.

An extract of fresh hyaline articular cartilage was prepared by mashing it up in distilled water, allowing the mixture to stand a short time, and then filtering it and using the clear solution. The same concentrations of salts were used here as in other cases, but the resulting precipitate was very much less in amount. Precipitation occurred only in the area of contact of the solutions, was in

a finely divided state, showed some tendency to form crystals, but mostly was in form of granules and very small spherules. There were no large spherules whatever. The total amount of deposit would lead one to think that very weak solutions had been employed instead of the usual ones.

Hyaline cartilage extract thus seems to decrease the amount of precipitation and to retain what deposit is formed in a very finely divided state. Addition of the various accessory substances used in previous cases had no effect, even lecithin apparently being powerless to produce the large spherules.

These facts may have some significance, for here we have an extract of a tissue which although apparently similar to other cartilage which does ossify, and although situated in continuity with ossified tissue, yet retains a matrix free from a deposit of calcium salts. Its extract forms the most unfavorable medium yet found in which to produce precipitation of calcium salts. Solutions of equal concentration in other media give three to four times the amount of precipitate found here. The experimental facts, it seems, are in complete accord with the nature of the tissue, which is unfavorable to the deposition of calcium salts in it.

Influence of Hydrogen-Ion Concentration.

Loeb's method was employed of treating gelatin to obtain it with a definite hydrogen-ion concentration. To 0.25 gm. gelatin in a test tube were added 20 c.c. of cold acid solution of a definite strength, and this was allowed to stand for 30 minutes, during which time the gelatin swelled remarkably, but did not dissolve. It was then filtered and washed on the filter paper four times with 25 c.c. of cold distilled water at 5° C. to remove all free acid.

The gelatin was then transferred to a test tube standing in hot water, where it melted, and the volume was made up to 25 c.c. with hot distilled water, thus giving a one per cent. gelatin solution. Two tubes for each strength of acid were prepared. On cooling the pH of each pair was determined approximately by Clark's colorimetric method, and then one tube of the pair was rendered a $M/10$ calcium chloride solution, and the other one a $M/4$ sodium carbonate solution, and these two solutions were placed as usual in a cell to react.

There was also an alkaline series made similarly to the above. In each series there were ten grades as follows:

Acid series treated with $\frac{1}{16}, \frac{1}{32}, \frac{1}{64} \cdots \frac{1}{8192}$ HCl.

Alkaline series treated with $\frac{M}{16}, \frac{M}{32}, \frac{M}{64} \cdots \frac{M}{8192}$ NaOH.

The pH of the acid series of washed gelatins in one per cent. solution ranged from 3.2 to 6.0. Untreated gelatin, both in sheets and in powder, which had been used for all the previous experiments was found to possess a pH of 6.0.

The weakest alkaline member of the series registered 6.2 and the fifth member of the series a pH of 9.0, above which no record was kept.

The amount of precipitate visible to the naked eye was less in the acid series than the alkaline one. It was least of all in those specimens where the pH was in the neighborhood of the isoelectric point of gelatin, which occurs in the acid series at 4.7, and which marks the point where the gelatin is most insoluble and most inert. In these slides there was practically no diffusion and spreading of the precipitate, only a narrow band of deposit occurring along the line of contact of the reacting solutions. Such a condition would lead one to infer that the salts in solution are intimately bound to the gelatin.

It was also noted that crystals (Figs. 17, 18, 19) were abundant, and very often large, in the various acid gelatins. Crystals were also present in the weaker alkaline specimens, but in gelatin treated with $M/2048$ NaOH or stronger alkaline solutions (Fig. 23) not a single crystal has yet appeared in a period of six months since the preparation of these slides. Here the precipitate is still in the form of very small spherules, often so densely packed as to prevent Brownian movement, but elsewhere still actively in motion, which has gone on continuously for six months without a change, and gives every indication of continuing indefinitely. The larger of these spherules, approaching one micron in diameter, have a slow movement and so can be well observed, and have given definite proof of what was already suspected from a study of other slides,

namely, that the so-called spherules are in reality flattened discs. During their vibratory movement many of them have been observed to slowly roll over and a narrow, thin edge is presented to the view instead of the circular outline. The appearance reminds one very strongly of a red blood corpuscle.

The largest and clearest spherules (Figs. 19–22) were formed in gelatin whose pH was between 4.7 and 7.5—that is, all in gelatin on the alkaline side of the isoelectric point—but in specimens whose reaction was either weakly acid or weakly alkaline, the neutral point being 7.0. This is the zone in which is obtained the formation of the largest spherules. Untreated gelatin, used for all the previous series of reactions without regard to hydrogen-ion concentration, has a pH 6.0 and so is within this zone. The pH of human blood and many tissues also lies in the alkaline part of this zone.

It is interesting to note that in strong acid members of the series precipitation is rapid, and while spherules may early be present, the deposit in the course of a short time becomes entirely crystalline in structure. In strongly alkaline members precipitation is very complete, but the deposit remains for an indefinitely long time in the form of small, discrete spherules, which do not grow or fuse, and which continue to exhibit Brownian movement. No crystals appear in these latter cases, even after the lapse of many months.

In the intermediate zone of weak acid, neutral, or weak alkaline gelatin both spherules and crystals appear in quantity, and the spherules are very large. In the part of this zone in which the hydrogen-ion concentration of blood and many tissues lies the deposit is mostly in the form of small spherules and the tendency to crystallization is very poor. This is essentially a colloidal phenomenon and seems significant in view of the fact that it has frequently been found that certain drugs in colloidal form have a much better therapeutic effect, have a much less damaging effect on healthy tissues, and are much more pleasant in their administration to the patient. As an example of this, compare the actions of silver nitrate and of argyrol, a colloidal silver vitellin. The colloidal preparation much more nearly approaches the condition of the tissues themselves and the reactions ought to be of a more col-

loidal character, which I have shown is the natural condition with the hydrogen-ion concentration on the basic side of the neutral point. To the acid side of this point reactions tend to the ordinary chemical crystalline variety.

Disintegration of Spherules.

A most remarkable phenomenon was observed during the last two months in some of the slides, where spherules have been observed in process of disintegration and transformation into crystals. This was first noticed in the acid gelatin series in a slide where the gelatin had a pH of 4.9 and where for the whole of six months appearance had been constant.

This case (Fig. 31) had offered one of the most perfect examples of large spherules, which were of perfect outline, glassy transparency, absolutely clear, and showing no indication at all of their internal structure. These spherules were formed in half an hour and had attained their full growth in less than two days, and for six months presented the same appearance, with no indications of a change. It happened at the end of this period that they were not examined for about one month, when it was discovered that some of them (Figs. 19 and 33) had disappeared, others were disintegrating, and all still present were opaque, while in this area which had not previously shown a single crystal there was now a number of large crystals.

Other slides were examined for similar conditions without avail, but a month later several of them showed signs of a change, and by examination of these at intervals a complete series of stages in the transformation has been obtained and a complete description of it can now be given.

The first sign of a change is in the occurrence of a very fine opaque central spot of a bluish tint (Figs. 20, 21, 24*a*, and 32) in the otherwise clear spherule. This area increases a little in extent, and the next stage then is ushered in by the appearance around this center (Fig. 24*b*) of a faint nebulous haze, which becomes more opaque and finally seems to consist of a fine radial striation (Fig. 24*c*) which extends farther and farther, until it reaches the periphery of the spherule, which is now an entirely opaque white

body. At this stage some spherules are uniformly opaque (Fig. 24*d*), others show a marked radial striation (Fig. 24*e*).

The final step in the change now occurs with a very gradual disappearance of the spherule, apparently by dissolving into the surrounding solution. It decreases in diameter and also in thickness until the remnant, diminished very much in size, appears like a nebulous ghost of the former spherule and eventually fades completely from view. During the solution the opaque center of many spherules seems to dissolve early (Figs. 24*f* and 32), converting the body into a ring.

In those cases where the spherule is uniformly opaque (Figs. 24*d* and *f*) solution is very uniform at the surface, the contour of the spherule remaining perfect while the size contracts. Where there is radial striation, however (Fig. 24*e* and *g*), the periphery dissolves unevenly, giving the spherule a rough, rather ragged surface.

Coincident with the beginning of the process of dissolving of the spherules, crystals occur in these areas which formerly were entirely composed of spherules, and which for several months' duration had not shown a single crystal. These crystals grow steadily both in size and in number (Figs. 19, 20, 21), while the spherules disappear. The passage of the material from the spherule into solution is evidently very transitory, it coming out again immediately to be deposited in the crystal.

What is the interpretation of this phenomenon? It seems to me that there is a very evident one, and that this is the same process very much delayed in starting, and very much protracted in its action, which occurs rapidly in ordinary aqueous solutions. It will be remembered that in precipitating calcium carbonate in pure water it is at first in a granular, amorphous form, which later redissolves and comes out of solution a second time as spherules and crystals, which grow rapidly, the spherules all being converted into crystals during growth.

Again in colloidal solutions the same process happens, but is delayed somewhat, taking hours or days instead of minutes. In such cases an area of spherules seen one evening after a precipitation, when examined next morning is found to consist of crystals.

Apparently in some of these cases at least, as Brownian movement ceases and the particles grow in size, the spherule is transformed directly into the crystal.

In the degeneration of the large spherule it seems to me we have a similar phenomenon to that of the other cases. The large spherules could correspond to the amorphous particles of the aqueous solution, fused into a large mass, the redissolving occurring very late to form the final crystal. There is evidently a very strong directive force toward the formation of crystals even in the colloids, and though interfered with and hindered by them for a long period, it nevertheless conquers. What it is that initiates the change after the deposit has remained stationary for many months is problematical. There was no infection in the gelatin, no change in its appearance, or any other fact to give a clue.

Some slides in other series in both gelatin and albumen also show evidence of some change in the spherules, which in view of the above facts may be expected to progress farther.

As far as I know this remarkable transformation of the precipitate has never been described before. The further investigation of this phenomenon is outside of my province and belongs to the field of physics and chemistry, where it will provide some very interesting problems which I hope some competent investigator will undertake to solve.

A second method of disintegration (Fig. 6) has also been observed in a lecithin solution, where some large spherules with very marked striation lost this striation after the lapse of several months and became dully opaque. Certain clear radii then appeared along which cleavage occurred, splitting the spherule into sectors which finally separated, giving triangular crystals.

Number of Forms Assumed by Precipitate.

It is to be remarked that in no slide was it possible, after Brownian movement ceased, to obtain only a single form of deposit. There were always two, and in many cases several forms occurring, and this applies equally to pure aqueous and to colloidal solutions. This multiplicity of form in the precipitate has also been noted by Biedermann and is due doubtless to differences in the relative con-

centration of the reacting salts and in the diffusion currents in different parts of the solution. Side by side may be found an area of crystals and of perfect spherules, or both forms may be seen mixed in one area.

In passing from the sodium carbonate end of a cell across the precipitate to the calcium chloride end there is often seen a marked change in shape of the deposit, forms present to one side of the line of contact of the two solutions not being present on the other. Spherules may occur all to the one side, but follow no rule as to which they select. Transition forms occur from one area to another. Orde found similar conditions on sectioning plugs of coagulated albumen with which he had sealed the ends of capillary tubes containing solutions which gave precipitates in the plugs when the sealed ends of the tubes were immersed in certain other solutions.

The multitude of forms exhibited in the precipitate of calcium carbonate in the slides seemed remarkable, but on referring to Goldschmidt's "Atlas der Krystallformen," I found illustrations for 2,544 modifications of the crystal form of calcite which have already been observed. Many of the changes are slight, but all are quite evidently different, and among them may be recognized all the types of crystal form I have obtained. Spherules and their variations were not shown.

PART 2. EXAMINATION OF BONE.

Technique and Material.

The ordinary method of examining bone by means of very thin ground sections, or by sections cut from decalcified bone, are useless to give the structure of the matrix. A method just published by Bast, however, for the study of bone cells *in situ*, without cutting sections, is equally applicable for the examination of the matrix and was here employed.

Bast worked with the parietal and ethmoid bones of mice and young rats, rabbits, cats and dogs, the nasal conchæ of these animals, pieces of the ethmoid in man, and other bones thin enough to be transparent. These were mounted whole. The specimens were fixed in 95 per cent. alcohol, washed in water, stained for

8 hours or more in diluted aqueous solution of Gentian violet, dehydrated in alcohol, cleared in benzol, and mounted in balsam.

To stain the bone cells only, the periosteum was left on the specimens, and this was later removed by dissection under a binocular microscope while the bones were in benzol. In this way the matrix remained clear, while if the periosteum was removed previous to staining, the matrix stained also.

This method was used by me, but as the matrix was the main object of study, most of my preparations were not stained, but were mounted for observation clear, in three different ways: first, without fixation, in a shallow cell filled with normal saline; second, in a shallow cell of glycerin; and third, fixed in 95 per cent. alcohol, then absolute alcohol, cleared in benzol, and mounted in balsam.

A cover glass to which some of the precipitate of calcium carbonate was adherent in the form of both spherules and crystals was passed through all the processes necessary to staining above and mounted in balsam. There was no change to be seen in the precipitate, so it can be inferred that calcium salts in the bony matrix, if in this form, would not be altered by any of the processes.

The specimens of bone used were parietal and ethmoidal bones and nasal conchæ of the mouse, frontoparietal bones of the frog, and splinters and thin sections in various planes from the femur, tibia, humerus, radius, ulna and scapula, of frog, mouse, guinea pig, dog, and human fœtus. The frog and mouse were young, so that their bones were in process of growth, and if there is any visible difference between new formed and old matrix it should be evident here. Thin pieces of the long bones cut with a sharp knife to take in areas of the total thickness of the shaft, or to show transition from the bony shaft to the cartilaginous epiphysis, were taken, splinters were clipped off long bones, small pieces were crushed, and all these were mounted as described above. The whole of a concha, scapula or parietal, if small, was mounted as one specimen.

In all cases the periosteum was carefully peeled off and was mounted alongside of the bone so that examination of it for newly formed matrix could also be made.

Results of Examination of Bone.

Both bright and dark field illumination were employed and specimens of bone, when thin, were quite transparent and easily examined. In bone that is well formed, whether from adult, or young individual, or fœtus, the appearances are similar. The bone cells and all their processes in the canaliculi are easily followed and appear as Bast has described them. The matrix round about them is homogeneous, transparent, and almost glassy in appearance, and gives no indication (Fig. 37) of being formed of separate particles. If it was originally formed in such a manner, there has been complete fusion, so that there is no evidence of a precipitation. In fact, the whole appearance bears out the statements of text-books of histology that, although two thirds of the bony matrix is composed of inorganic salts, they are so intimately blended with the organic material that there is no visible evidence of their presence.

In the case of growing long bones, such as those of the limbs, sections were taken across the shaft to include the newly formed growing periphery just under the periosteum, and also sections in the length of the bone to include the end of the diaphysis, the epiphyseal plate of cartilage, and the bony epiphysis. These sections, however, were disappointing, as even at the transitional areas any tissue that could be identified as bony matrix appeared homogeneous, so that no evidence of precipitation could be obtained here.

In the case of the developing diaphysis of the long bones of the limbs in human fœtuses, however, there were definite, discrete particles to be seen. In Quain's Anatomy there is a fine description by Schäfer of the appearances of developing bone with which my findings entirely agree. Just ahead of the advancing bone of the diaphysis is an area of change, where the tissue is no longer cartilage, but is not yet true bone. Here we find definite fibres (Fig. 38) like those of white fibrous tissue, and known as Sharpey's fibres, extending from the bony material forward into the cartilage, and in these fibres in the narrow area immediately in advance of the true bone are numerous very fine granules which appear similar to the material of the matrix in well-formed bone and so are evidently the bone salts. They are crowded in the fibres just ahead of the actual bone, and in the bone itself we find

the homogeneous appearance due to the fusion of these discrete particles into one mass. Pacchioni has also described the first appearance of lime salts in bone in the form of fine granules which later form a homogeneous mass.

The possible origin of these particles either by precipitation or secretion will be discussed later.

PART 3. DISCUSSION OF RESULTS.

Concerning some of the physical phenomena of the precipitation reactions studied, remarks have been made at appropriate places in the previous pages describing the work and need not be repeated here.

There are a few points to be constantly borne in mind in considering the question of ossification, which have been very clearly demonstrated by Wells. The most important is the fact that there is a very definite and fixed ratio between the amount of calcium carbonate and calcium phosphate deposited in bone. The second point is that this same ratio is equally true in areas of calcification in tissues other than bone. The third fact is that the processes of ossification and of calcification are essentially the same, the kind of matrix or tissue in which the deposit occurs differentiating the two processes.

Theories Regarding Deposition of Calcium Salts.

The reactions with calcium phosphate show it to be invariably precipitated in a granular form so that if it were visible in bone it should be evident in a finely divided amorphous form. As this salt forms over eighty per cent. of the inorganic material, it ought to be readily demonstrated, but this is not the case, because the matrix is clear and homogeneous, not granular, except at the advancing edge of ossification in developing fœtal bones.

It has been shown that in mixed solutions calcium carbonate is not prevented, by the presence of the phosphate, from coming out in typical spherules or crystals, so that it might be expected that the carbonate would show in one of these forms in bone, in which it forms fifteen per cent. of the salts. This expectation is further strengthened by the fact that typical spherules have been found in the shells of invertebrates, which are mostly calcium carbonate, by

Rainey and by Biedermann. The dentine and enamel of the teeth, Rainey believed he had demonstrated to be composed of spherules, forming in rows and coalescing. Neither in old bone nor in new, nor in a transitional area transforming from cartilage to bone, have I seen either a single crystal or a single spherule.

Previous work has demonstrated that spherules can always be found in calcium carbonate precipitated in colloids. This formation is favored by the occurrence of the reaction gradually and slowly, and also by the presence of lecithin, which is found in both blood and bone, so that all the conditions in the matrix of the bone are favorable to the formation of spherules if precipitation occurs. The hydrogen-ion concentration is also favorable to formation of large spherules. With optimum conditions for their occurrence, and with a complete absence of these bodies, the interpretation is that the salts found in the bony matrix are not deposited in it by simple precipitation from a double decomposition of salts in the blood or the tissue. This is purely negative evidence, which, of course, is not of the same value as positive evidence, but it seems to me forms a very strong point against the simple precipitation theory.

It must be noted, however, that if calcium carbonate is very small in amount compared to the amount of phosphate precipitated that spherules may not be in evidence, but the whole precipitate will be granular, with coarser granules, resembling minute spherules and interpreted as carbonate interspersed among the masses of finer granules formed by the phosphate. It must be emphasized that homogeneous masses such as seen in the bony matrix never occurred in any of my experiments, but precipitates, no matter how long they were kept, remained composed of distinct discrete particles.

Another point against this theory is that it supposes that a soluble salt of calcium circulates in the blood to be precipitated as phosphate and carbonate in the bone. With the large amount of sodium carbonate and sodium phosphate in the blood, calcium carbonate and phosphate should be formed at once, here and not in the bone. But calcium is present in the blood in a greater amount than calcium carbonate and calcium phosphate are soluble in water.

And so it might appear that calcium can not be carried in the blood in the form in which it is later deposited in the bone. Pauli and Samec, however, have shown that difficultly soluble salts like calcium carbonate may have the amount of their solubility in water increased to over seven-fold in an albuminous solution, and so it is possible that the bone salts, formed by interaction of the blood salts and calcium absorbed into the blood as calcium chloride, might still be carried in solution in the blood and deposited in the bone.

This is closely related to the views of Barillé, whose theory is a step in advance of this, and is strongly supported by such work as that done by Wells. According to this view the calcium is in the blood in the form of a double salt formed here, tribasic calcium carbonophosphate, $P_2O_8Ca_2H_2 : 2CO_2(CO_3H)_2Ca$. This is soluble in the blood concentration of carbon dioxide. Infiltrating tissues like the bony matrix where the carbon dioxide content is lowered, this is precipitated and immediately breaks up into calcium carbonate and dicalcium phosphate, which latter salt is converted into calcium phosphate, giving the final proportion found in bone of 15 parts calcium carbonate to 85 parts phosphate. Even by this method of precipitation, however, one might expect to be able to see the salts in the matrix, and not being able to do so argues in favor of their being combined with the matrix. The most important point brought out in this theory is a clear consistent explanation supported by experimental evidence, as to the fixed proportion in the amount of the two calcium salts, the carbonate and phosphate, which is always found in their deposition.

It is interesting to note here the following facts regarding cartilage. Wells has shown that there is an almost specific power in cartilage for the absorption of calcium salts. My work is in agreement with this, for in cartilage extracts there was obtained the least amount of precipitation obtained in any of the experiments, thus showing the power of the substances in cartilage to hold on to the calcium. In spite of this affinity for calcium, however, microscopic evidence of developing ossification in bones preformed in cartilage shows us that the deposition of calcium is never directly in a cartilaginous matrix, but that the cartilage preceding the bone is destroyed and replaced by the organic bony matrix which is of

a fibrous character, and this then has the calcium salts deposited in it.

The final possible explanation of the deposit of bone salts in the matrix is the secretory one. Wells and others have been forced to the conclusion that there is a selective or specific action of tissues, such as cartilage or membrane about to ossify, by which calcium salts are absorbed or otherwise taken into them, while apparently entirely similar areas of cartilage and membrane do not ossify at that time or at any later time. From this view of selective action it is not a far step to that of secretory action, explaining the ossifying process as a secretion of the calcium salts by the bone forming cells. It is already generally admitted that the matrix is a product of the bone cells, and if this is true, it is quite logical to assume that the calcium salts are taken by the bone cells from the blood and passed on into the matrix.

The difficulty in this theory has been to account for the amount of phosphate in the tissues. The calcium was looked upon as being derived from that carried in the blood, but the phosphate was explained as being due to cellular activities, derived either from the nucleus or from cellular degeneration. Another difficulty here, again, is to explain the fixed ratio of carbonate and phosphate. This, of course, could be looked upon as due to the carbon dioxide content of the tissue, or as due to the activity of the cell, a definite proportion being found in the secretory products here just as in other organs of the body.

It seems to me, however, that the nearest approach to the truth is to be obtained by combining two views. The bone salts, I believe, reach the tissue as the soluble double salt, tribasic calcium carbonophosphate, which by its constitution provides for the tissue both calcium carbonate and calcium phosphate, each salt in the proper proportion found in bone. I do not believe, however, that deposit here is by precipitation, for in the bone, owing to the colloidal matrix, the hydrogen-ion concentration, and the presence of lecithin, all conditions are most favorable to a visible and characteristic precipitate, showing granules and spherules, but there is no visible evidence whatever of these bodies. The only evidence in favor of precipitation consists of the following two sets of facts:

First, Schäfer and Pacchioni have described the first appearance of bone salts in developing bone in the form of minute granules which quickly coalesce. This I have confirmed. This evidence could also be interpreted as showing the presence of the salts to be due to secretory activity. Secondly, Olivier in a tumor of the breast and Pettit in a tumor of the maxilla and also in a renal cyst saw and made illustrations of calcospherites. These bodies can be explained only as a precipitation phenomena, but they do not afford sufficient evidence to prove that this is the process whereby the bone salts are deposited. And so these salts, after reaching the bone in correct proportions as the double salt above mentioned, must be deposited in the matrix by some more subtle process than ordinary precipitation.

Here we have recourse to the secretion theory. The bone salts in their proper proportions having been brought to the cell by the blood, are taken by the cell and secreted as an integral part of the matrix in combination with the ossein, the organic base, which it is generally admitted already, is a product of the cells. This will produce a matrix uniform in appearance, with salts invisible, and provides the proper proportion of each salt, as the cell is in its turn provided with the two salts in that exact amount. Histological evidence shows that the fibrous matrix appears first in the newly forming bone and impregnation with the bone salts begins immediately, the salts showing as fine granules at first, and coalescing as their quantity increases, to form a homogeneous matrix.

Reversibility of Calcium Reaction.

There seems to be evidence that this process of secretion is reversible, the bone cells being able to take up the calcium salts again. Wells and others have shown that calcium is removed from the bones in cases where there is a great demand for it in the body, as in pregnancy, or where it is being steadily lost by the body, for instance by way of a pancreatic fistula. Also, the osteoclasts responsible for the normal resorption of bone are supposed by some authors to be only osteoblasts which have changed their function. And further, where a graft of either living or dead bone, or a bone plate and bone screws are used to repair an injury, a process known

as creeping replacement occurs whereby this material is gradually absorbed and replaced by new bone. It has been shown by Gallie and Robertson that it is the osteoblasts which invade the graft, gradually absorb the old matrix and bone salts immediately surrounding them, and then replace this by newly formed substance.

If the bone salts were laid down by precipitation of tribasic calcium carbonophosphate due to a change of the carbon dioxide content, it seems to me it would be impossible for this precipitate to be removed again as easily as Wells states it is, in response to the body's need for calcium. Would pregnancy or a pancreatic fistula so change the carbon dioxide content of the bone, as to make possible a reversal of the conditions that brought about the precipitation, and so either provide for increased solubility of the two salts or else make possible the recombination of the carbonate and phosphate of calcium into the double salt, with its consequent solution and carrying off in the blood?

It seems to me that the carbon dioxide content in the bone will not vary enough to permit of such a reversal, as to raise it would indicate a high activity in a tissue whose metabolism is low. This low-grade activity is given as a reason for obtaining the precipitate.

The taking of the calcium salts out of the bone probably depends on some balance that has to be maintained between the blood, the osteoblast, and the bony matrix. If the blood is rich in calcium, the cell can take it and pass it on into the matrix. If the blood is poor in calcium, the cell to maintain its relation in balance to the blood takes from the matrix to add to the blood. This is an instance comparable to the relations between the blood and its dextrose, and the liver cell and its stored glycogen, to maintain a certain carbohydrate balance.

This view also follows logically upon that of Wells, who regards the bony matrix as a great reserve store of alkaline bases for the body, where the calcium salts are in a state of flux, being constantly added to or drawn upon according to the needs of the body. Changes in the bones are much greater in extent and more rapid in their occurrence than is generally believed. It seems to me that this idea of the reversibility of the direction in which calcium salts

pass through the tissues is a most valuable one for the proper comprehension of some of the processes of growth, repair of fractures, and changes going on in rarefaction of bony tissues.

Summary.

1. Calcium phosphate precipitated in water or in colloidal solutions is constantly granular and amorphous in character, and apparently uninfluenced by the nature of the solution.

2. Calcium carbonate precipitated in water shows a great diversity of crystalline form; in colloidal solutions it shows two main forms, an irregular crystalline one and a spherical.

3. Mixtures by simultaneous precipitation of both salts in the same solution show each salt separating out independently and the part of the deposit formed by each can be easily identified.

4. Spherule formation by calcium carbonate is a typical reaction in colloidal solutions.

5. Spherules and crystals are influenced in shape, size, number, and internal structure by a variety of substances found normally or pathologically in the blood, most notably by lecithin which favors the formation of large spherules, and by acetone which increases the rapidity and extent of precipitation.

6. The character of the deposit of calcium carbonate is influenced by the hydrogen-ion concentration of the colloidal solution, being most crystalline in acid media, all in the form of spherules in strongly alkaline media, and mixed in form in solutions neutral or nearly neutral.

7. Large spherules, after persisting for months may undergo a sort of degenerative process, with change of internal structure, after which they dissolve and the material forming them is laid down in a crystalline form. As far as I know this phenomenon has never been previously described.

8. In fresh bone of various animals examined in various ways there is no visible microscopic evidence of the bone salts, although they form two thirds of the mass of the matrix. The inference is that the bone salts are not deposited in the matrix by simple precipitation, for the conditions are such that, if precipitated, granules, spherules, and crystals should be visible.

9. In the rapidly developing fœtal skeleton the first appearance of bone in the matrix is in the form of fine granules or globules which quickly fuse or coalesce to form a homogeneous mass. This might be interpreted according to the bias of the observer as supporting evidence either of precipitation or of secretion of the salts into the matrix.

10. The view advanced by Barillé and supported by Wells's work, that calcium is carried in the blood as tribasic calcium carbonophosphate, is probably correct, as it furnishes the bone salts in the proper proportion, but their view of its deposit in the matrix as a precipitate due to change in concentration of carbon dioxide does not appear correct in view of the fact that no precipitate of the bone salts is visible.

11. The theory that the salts furnished by the blood are taken by the bone cells and secreted by them along with the matrix seems reasonable in view of the condition found in the matrix.

12. The action of the osteoblasts seems to be reversible, they being able to absorb or take up the calcium salts again out of the matrix.

In concluding this paper I wish to acknowledge my indebtedness to others by thanking Professor J. Playfair McMurrich for special apparatus and for valuable references, also the following for providing material for investigation: Professor W. H. Piersol, Dr. R. D. Defries, Dr. Banting, Dr. Simpson, Dr. Dafoe, and Dr. Low.

BIBLIOGRAPHY.

Barillé, A.

'04 De l'action de l'acide carbonique sous pression sur les phosphates métalliques; combinaison (carbonophosphates) ou dissolution, applications diverses. Journ. de Pharm. et de Chimie, Ser. 6, T. 19, p. 14, 71, 196, 245, 295.

'10 Rôle des constituants de la dissociation de la carbonophosphate tricalcique dans la genèse du tissu osseux et des diverses concrétions à base de phosphate et de carbonate de calcium. Journ. de Pharm. et de Chimie, Ser. 7, T. 1, p. 342.

Bast, T. H.

'21 Studies on the Structure and Multiplication of Bone Cells facilitated by a new Technique. Amer. Jour. of Anat., Vol. 29, pp. 139–157.

Biedermann. W.

'02 Ueber die Bedeutung von Krystallization prozessen bei Bildung der Skelette wirbelosser Tiere, namentlisch der Molluskenschalen. Zeitschr. für allgemeine Physiol., Bd. I., S. 154–208.

'02 Untersuchungen über Bau und Entstehung der Molluskenschalen. Jenaische Zeitsch. für Naturwiss., Bd. XXXVI., S. 1–164.

Bowman J. H.

'06 A Study in Crystallization. Journ. of Soc. of Chem. Indust., Vol. 25, p. 143.

Clark, W. M.

'21 The Determination of Hydrogen Ions. Williams and Wilkins Co., Baltimore.

Gallie, W. E., and Robertson, D. E.

'18 The Transplantation of Bone. Journ. of Amer. Med. Ass., Vol. 70, pp. 1134–1140.

'19 The Repair of Bone. Brit. Journ. of Surg., Vol. VII., No. 26, pp. 211–216.

Goldschmidt, V.

'13 Atlas der Krystallformen. Vol. 2 and Atlas Vol. 2. Winters, Heidelberg.

Gradwohl, R. B. H., and Blaivas A. J.

'20 The Newer Methods of Blood and Urine Chemistry. 2d Edition. C. V. Mosby Co., St. Louis, U. S. A.

Harting, —.

'72 On the Artificial Production of some of the Principal Organic Calcareous Formations. Quart. Journ. Micros. Science, New Series, Vol. XII., pp. 118–123.

Holmes, H. N.

'17 The Formation of Crystals in Gels. Journ. of Physical Chem., Vol. XXI., pp. 709–733.

Lillie F. R.

'08 The Development of the Chick. Henry Holt and Co., New York.

Link, H. F.

'39 Ueber die erste Entstehung der Krystalle. Annalen der Physik und Chemie. Poggendorf, Bd. 46, S. 258.

Loeb, Jacques.

'18 Amphoteric Colloids, I.–V. Journ. of Gen. Physiol., Vol. I. I. Chemical Influence of the Hydrogen Ion Concentration, p. 39. II. Volumetric Analysis of Ion Protein Compounds; the Significance of the Isoelectric Point for the Purification of Amphoteric Colloids, p. 237. III. Chemical Basis of Influence of Acid upon the Physical Properties of Gelatin, p. 363. IV. The Influence of the Valency of Cations upon the Physical Properties of Gelatin, p. 483. V. The Influence of the Anions upon the Physical Properties of Gelatin, p. 559.

Loeb, Jacques.

'20 Ion Series and the Physical Properties of Proteins. Journ. of Gen. Physiol., Vol. 3, p. 85.

'20 The Proteins and Colloid Chemistry. Science, New Series, Vol. LII., No. 1350, pp. 449–456.

Olivier, E.

'95 Cancer gelatineux du Sein avec Corps Calcaires. Beitr. zur Path. Anat. und zur Allgem. Pathol., Bd. XVII.

Ord, W. M.

'72 On Molecular Coalescence and on the Influence of Colloids upon the Forms of Inorganic Matter. Quart. Journ. Micros. Science, Vol. XII., pp. 219–239.

Pacchioni D.

'02 Untersuchungen über die normale Ossification des Knorpels. Jahrb. f. Kinderheil. Berl., Bd. LVI., S. 327–340.

Pauli. W., und Samec, M.

'09 Ueber Löslichkeitsbeeinflussung von Electrolyten durch Eweisskörper. Biochem. Zeitschr., Bd. 17, S. 235–256.

Pettit, A.

'97 Sur le Rôle des Calcospherites dans la Calcification a l'État Pathologique. Arch. d'Anat. Micros., T. 1, p. 107.

Rainey, George.

'57 On the Formation of the Skeletons of Animals, and other Hard Structures formed in Connection with Living Tissues. Brit. and Foreign Med. Chir. Review, Vol. XL., p. 343.

'58 Precise Directions for making of Artificial Calculi, with some Observations on Molecular Coalescence. Quart. Journ. Micros. Sci., Vol. VI., pp. 41–50.

'59 On the Structure and Mode of Formation of the Dental Tissues, according to the Principle of Molecular Coalescence. Quart. Journ. Micros. Sci., Vol. VII., pp. 212–225.

'61 Some Further Experiments and Observations on the Mode of Formation and Coalescence of Carbonate of Lime Globules, and the Development of Shell Tissues. Quart. Journ. Micros. Sci., New Series, Vol. 1, pp. 23–32.

Richards T. W., and Archibald, E. H.

'01 A Study of Growing Crystals by Instantaneous Photomicrography. Amer. Chem. Journ., Vol. XXVI.

Schäfer, E. A.

'12 Text-book of Microscopic Anatomy. Quain's Elements of Anatomy, Eleventh Edition, Vol. II., Pt. I. Longmans, Green and Co., London and New York.

Wells, H. G.

'06 Pathological Calcification. Journ. Med. Research, Vol. XIV. (N. S., Vol. 9), p. 491.

'10 Calcification and Ossification. Harvey Lectures, 1910–11.

'11 Calcification and Ossification. Arch. of Int. Med., Vol. VII., p. 721.

'18 Chemical Pathology. Third Edition. W. B. Saunders and Co., Philadelphia and London.

EXPLANATION OF PLATES.

PLATE I.

All figures are from camera lucida tracings and are of equal magnification, × 200, so that the relative size of precipitated particles in each case can be compared. Only forms which were common, and appeared in quantity have been shown.

All illustrations are to show forms assumed by calcium carbonate precipitated from various solutions by the reagents listed below.

FIG. 1. Crystals of calcium carbonate precipitated by sodium carbonate in distilled water.

FIG. 2. Crystals of calcium carbonate precipitated by ammonium carbonate in distilled water.

FIG. 3. Crystals of calcium carbonate precipitated by potassium carbonate in distilled water.

FIG. 4. Deposit of calcium carbonate precipitated by sodium carbonate in distilled water containing 0.1 per cent. lecithin. Note small spherules, and small crystals, a tendency towards clumping, a very large spherule and a large rosette.

FIG. 5. Deposit of calcium carbonate precipitated by ammonium carbonate in distilled water containing 0.1 per cent. lecithin. Spherules of various sizes, a marked tendency to fusion, and formation of large irregular masses are to be noted.

FIG. 6. Deposit of calcium carbonate precipitated by potassium carbonate in distilled water containing 0.1 per cent. lecithin. Note a tendency to clumping, various sizes of spherules with some double forms. The last three forms indicate one process of disintegration of a spherule, by the occurrence of radial lines along which it splits into sectors, which thus become triangular crystals.

FIG. 7. Deposit of calcium carbonate precipitated by sodium carbonate in a solution of egg albumen. Practically the whole precipitate was in form of spherules.

FIG. 8. Deposit of calcium carbonate precipitated by ammonium carbonate in a solution of egg albumen. Spherules of varying size are seen, also some crystals.

FIG. 9. Deposit of calcium carbonate precipitated by potassium carbonate in a solution of egg albumen. Many spherules are seen, and a tendency towards fusion is noted.

FIG. 10. Deposit of calcium carbonate precipitated by sodium carbonate in a solution of egg albumen containing 0.1 per cent. lecithin. Note the markedly increased size of spherules and crystals and the tendency to fusion.

FIG. 11. Deposit of calcium carbonate precipitated by ammonium carbonate in a solution of egg albumen containing 0.1 per cent. lecithin. Note the increased size of particles, and their tendency to fuse.

FIG. 12. Deposit of calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 1 per cent. of acetone. Crystals and spherules both occur. One crystal is seen fused with a spherule.

FIG. 13. Deposit of calcium carbonate by interaction of calcium chloride in a 1 per cent. gelatin solution with sodium carbonate in a 4 per cent. gelatin.

Fields are shown by various sized spherules, of crystals, and of spherules and crystals mixed.

FIG. 14. Deposit of calcium carbonate by interaction of calcium chloride in 1 per cent. gelatin solution with ammonium carbonate in 4 per cent. gelatin solution. Spherules of various sizes are seen.

FIG. 15. Deposit of calcium carbonate by interaction of calcium chloride in 1 per cent. gelatin solution containing 0.1 per cent. lecithin, with ammonium carbonate in 4 per cent. gelatin solution containing 0.1 per cent. lecithin. Note the increase in size of the spherules over those shown in Fig. 14. Three different degrees of fusion are illustrated in the double forms.

FIG. 16. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of untreated gelatin.

FIG. 17. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $\frac{1}{16}$ HCl solution. Large crystalline precipitate.

FIG. 18. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $\frac{1}{32}$ HCl solution. Large, markedly crystalline deposit.

FIG. 19. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $\frac{1}{512}$ HCl solution. The field shown in this figure was composed for some months altogether of clear spherules and a few dumb-bells. The spherules here illustrated are now undergoing disintegration and in their place are appearing large crystals. Some debris is seen in one corner. One spherule is seen partly from the side, showing its true shape to be a disc.

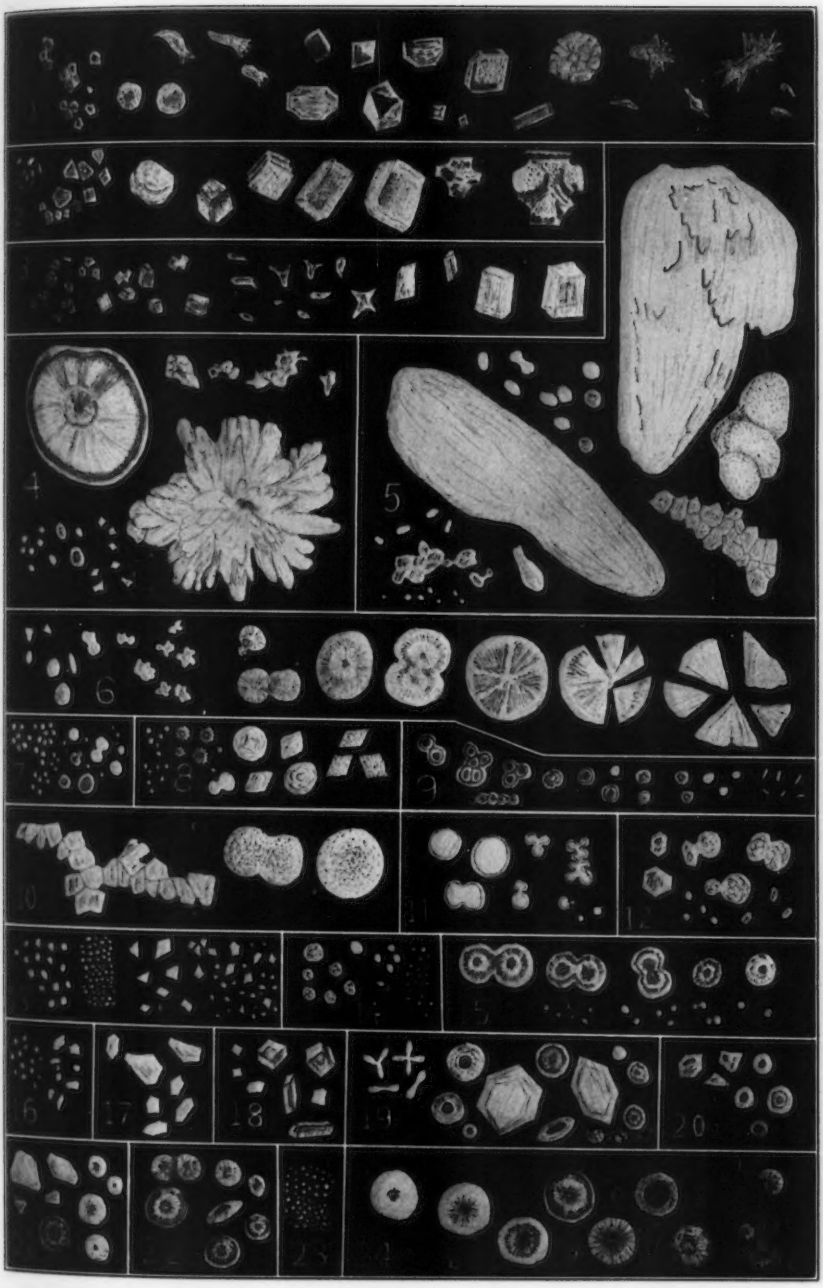
FIG. 20. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $\frac{1}{2048}$ HCl solution. Disintegrating spherules and new crystals are seen.

FIG. 21. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/8192$ NaOH solution. Newly formed crystals and various stages of disintegrating spherules are shown.

FIG. 22. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/4096$ NaOH solution.

FIG. 23. Deposit of calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with $M/2048$ NaOH solution. Precipitate is all in the form of small spherules which have been in constant Brownian movement for six months.

FIG. 24. A series of spherules showing various stages in the disintegrative changes which occur before they redissolve. *a*, Spherule with small central opaque area. *b*, Spherule with opaque radial striation extending from central spot. *c*, Spherule with further extension of opaque area. *d*, Spherule almost completely opaque. Narrow, bright, clear periphery. *e*, Spherule striated right out to periphery. *f*, Spherule like that in *d*, dissolving both at periphery and in center in a regular manner. *g*, Spherule like that in *e*, dissolving at an irregular rate at the periphery. *h*, *j*, Remains of dissolving spherules, and debris of some that have fallen apart.



J. C. WATT.

PLATE 2.

Photomicrographs all taken at a standard magnification, × 200, with dark field condenser in use.

FIG. 25. Crystals of calcium carbonate precipitated by ammonium carbonate in water.

FIG. 26. Calcium carbonate precipitated by sodium carbonate in a solution of egg albumen containing *N*/10 sodium chloride.

FIG. 27. Calcium carbonate precipitated by potassium carbonate in water containing 0.1 per cent. lecithin. Note one double spherule and three large single spherules of which the two larger show indications of the radial divisions which will result in splitting the spherule into crystals of triangular outline. Many small crystals and spherules seen.

FIG. 28. Calcium carbonate precipitated by ammonium carbonate in water containing 0.1 per cent. lecithin.

FIG. 29. Calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 1 per cent. ethyl alcohol.

FIG. 30. Calcium carbonate precipitated by potassium carbonate in a solution of egg albumen containing 0.05 per cent. urea.

FIG. 31. Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/1024 HCl solution. Clear spherules, some seen from side appearing disc-like.

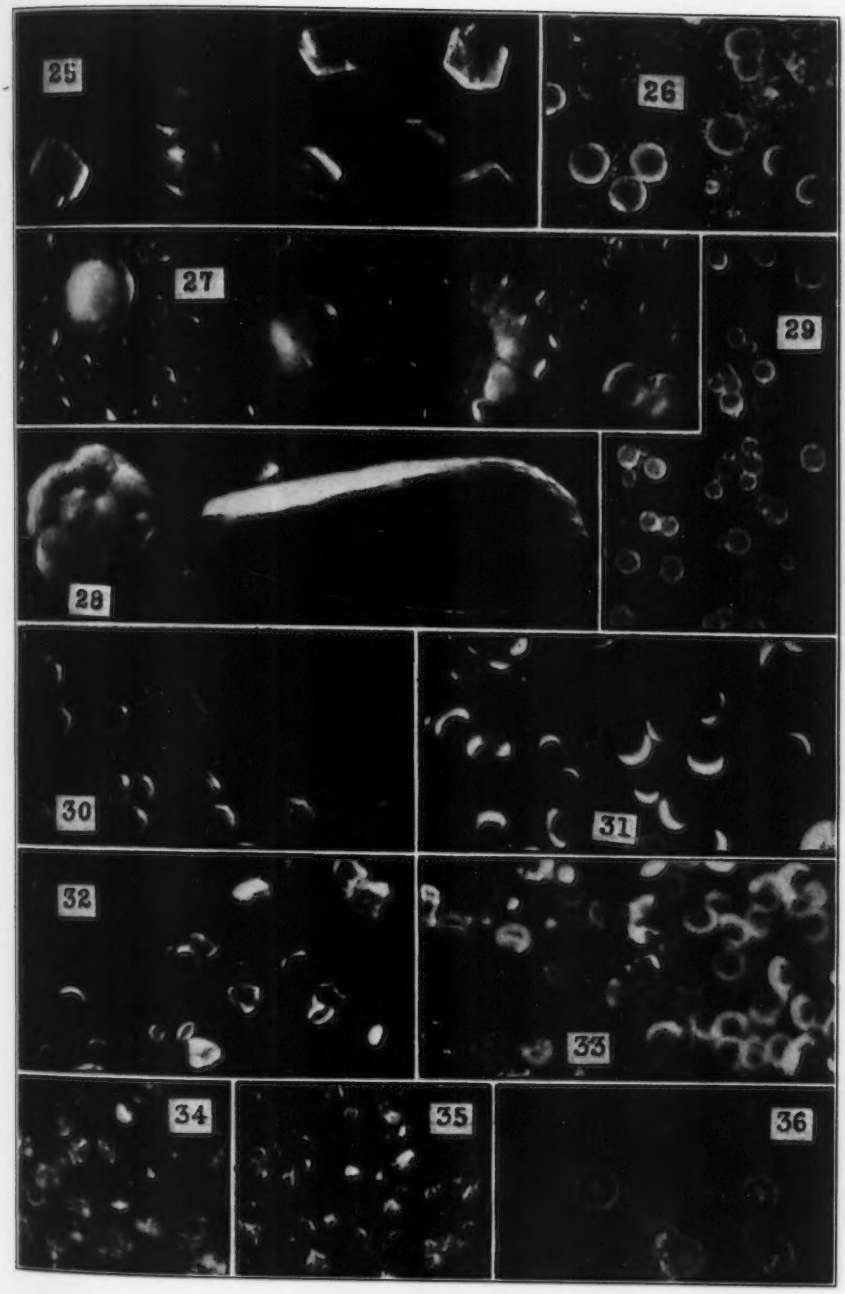
FIG. 32. Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/2048 HCl solution. Some crystals are present. Spherules begin to show an opaque central dot, the first indication of a coming disintegration.

FIG. 33. Calcium carbonate precipitated by sodium carbonate in a 1 per cent. solution of gelatin previously treated with 1/512 HCl solution. This field was formerly all spherules, now some crystals are seen. Spherules are far advanced in disintegrative process, are completely opaque, and are dissolving, some appearing only as ghosts of their former selves.

FIG. 34. Calcium carbonate precipitated by ammonium carbonate in 1 per cent. gelatin solution containing 0.05 per cent. urea. Rings and striations seen in spherules.

FIG. 35. Calcium carbonate precipitated by sodium carbonate in a 1 per cent. gelatin solution containing 0.05 per cent. urea. Field of very small mixed crystals and spherules.

FIG. 36. Combined precipitate of calcium phosphate and calcium carbonate by action of sodium phosphate and sodium carbonate in dog's blood serum. The calcium carbonate shows as seven spherules. The calcium phosphate shows as a cloud of very fine amorphous granules, which is the form in which it precipitated in all experiments.

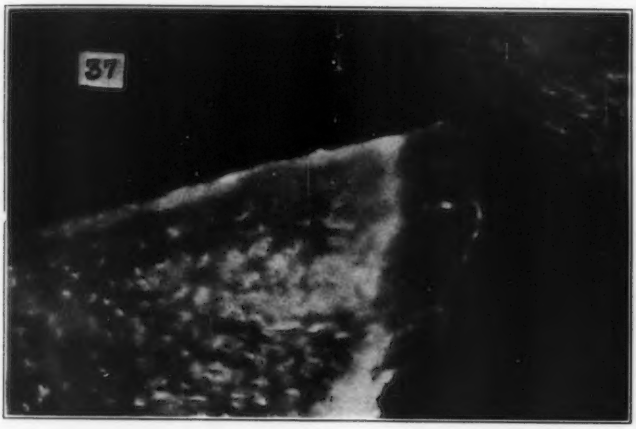


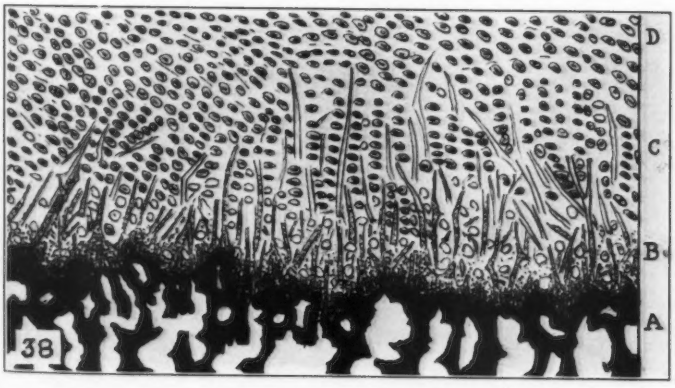
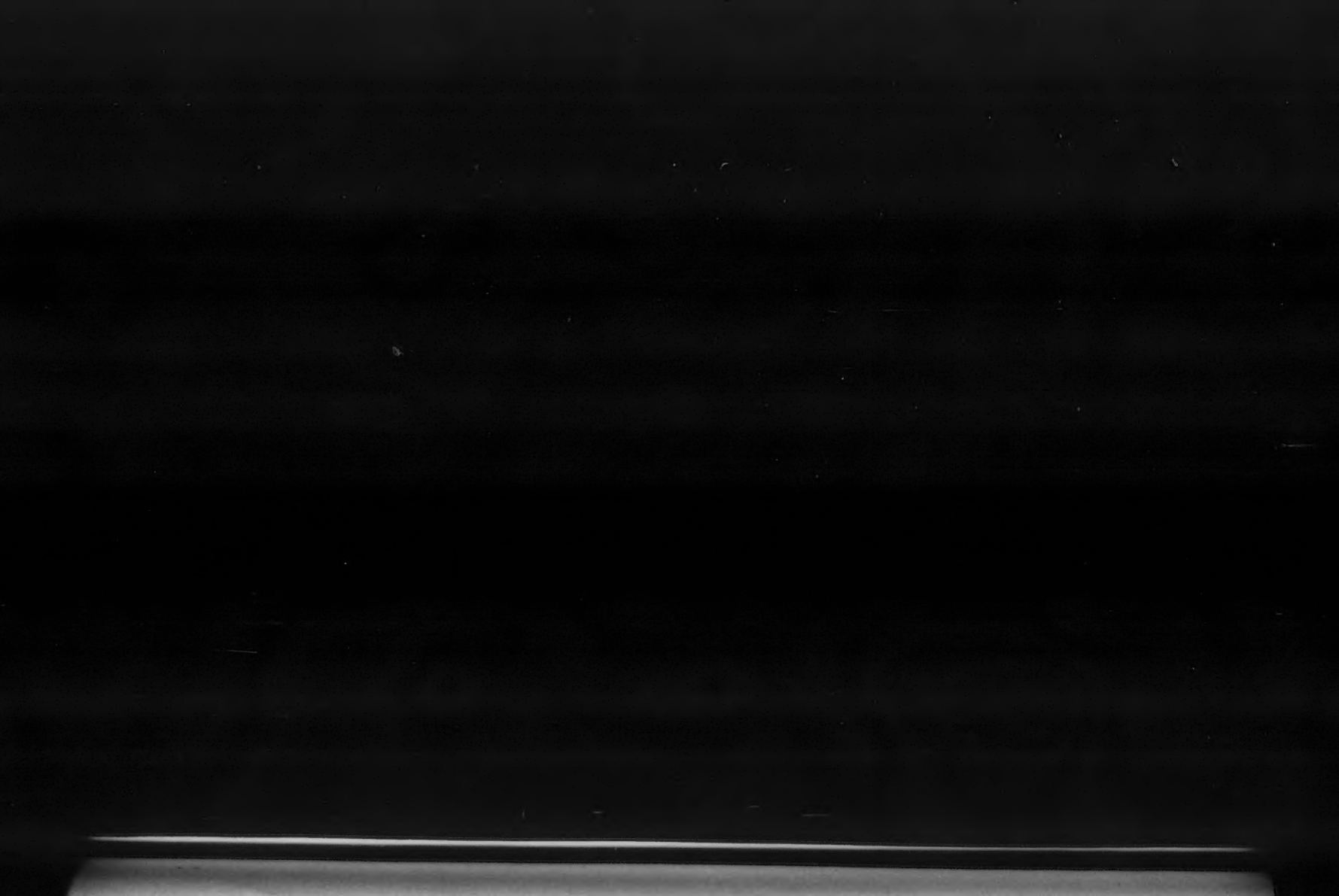
J. C. WATT

PLATE 3.

FIG. 37. Photomicrograph of a small piece of bone from the growing femur of a mouse. The bone cells show as small, oval, opaque bodies in the specimen. No discrete particles can be seen in the matrix. Magnification, × 200.

FIG. 38. Outline sketch made with camera lucida, of the growing bony end of the diaphysis of the humerus of a six months human foetus. Four zones can be distinguished. *A*, fully formed bone spicules (shown as solid black, marrow spaces white, bone and marrow cells not indicated) with calcium salts fused. *B*, osteogenic fibres, impregnated with calcium salts in form of minute granules. *C*, newly forming osteogenic fibres, not yet impregnated with bone salts, and cartilage showing changes preparatory to bone formation. *D*, unaltered cartilage. Magnification, × 300.





J. C. WATT.